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The Journal of Clinical Investigation

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Babson, A. L., Read, P. A., and Phillips, G. E.: The importance of the substrate in assays of acid phosphatase in serum. Am. J. of Clin. Path. 32:1 83-87, July 1959.

A comparison is made between six generally used substrates for their relative specificity to prostatic and erythrocytic acid phosphatase. Results showed that a new substrate, alpha-naphthyl phosphate, was twice as specific as beta-glycerophosphate and 40-100 times as specific as all other substrates investigated. Acid phosphatase assays in serum should be specific for that enzyme arising from cancerous prostatic tissue. The substrate alpha-naphthyl phosphate* is specific for this enzyme.

Babson, A. L., and Read, P. A.: A new assay for prostatic acid phosphatase in serum. Am. J. Clin. Path. 32:1, 88-91, July 1959.

A new and specific method for the determination of prostatic acid phosphatase in serum is presented. The method is based on the use of a new substrate alpha-naphthyl phosphate which measures only prostatic acid phosphatase. The method is simple and rapid to perform with a minimum of manipulation. In this procedure the color developed represents prostatic enzyme activity alone. The reagents for this new method are available in convenient and stable form.*

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(1) Matthias, J. Q.; Misiewicz, J. J., and Scott, R. B.: Brit. M. J. 2:1837-1840 (Dec. 24) 1960.
(2) Coggins, P. R.; Ravdin, R. G., and Eisman, S. H.: Cancer *13*:1254-1260 (Nov.-Dec.) 1960.
(3) Papac, R.; Petrakis, N. L.; Amini, F., and Wood, D. A.: J.A.M.A. *172*:1387-1391 (March 26) 1960.

DOSAGE: *For neoplasms relatively susceptible to Cytoxan* — Patients with lymphomas and other neoplasms believed to be relatively susceptible to Cytoxan therapy are given an initial dose of 2-3 mg./Kg./day intravenously. White blood counts and platelet determinations should be made daily or twice weekly and the dosage adjusted accordingly. Intravenous infusions should be continued for at least 6 days unless otherwise indicated. A leukopenia of between 1500 and 5000 cells per cu. mm. (or lower) may be expected between the tenth and fourteenth day. In the presence of a leukopenia of less than 2000/cu. mm. Cytoxan should be discontinued until the white cell count returns to 2000 to 5000 (usually within a week). Dosage is subsequently adjusted as indicated by the patient's objective response and the leukocyte count. If the patient is subjectively improved, if the size of the tumor has decreased, or if the white cells are satisfactorily maintained between 2000 and 5000/cu. mm. oral dosage may be instituted equivalent to intravenous dosage.

Thrombocytopenia is rarely observed on this regimen. If platelet counts of less than 100,000/cu. mm. are observed, the patient should be watched carefully. If platelets continue to decrease, Cytoxan should be discontinued.

The patient who has had previous treatment with alkylating agents, or x-ray, or is debilitated may be more susceptible to bone marrow depression, and initial Cytoxan doses should be more conservative than the above. Such patients should have more frequent hematologic evaluation. Good medical practice demands access to a reliable hematologic laboratory when using Cytoxan.

For neoplasms relatively resistant to Cytoxan — Patients with carcinomas and other malignant neoplasms believed to be less susceptible to Cytoxan therapy are given a dose of 4 to 8 mg./Kg./day intravenously. Unless there are indications to the contrary, this dose is continued for 6 days, then stopped. Leukopenia usually ensues on the tenth to fourteenth day after the first dose of Cytoxan. Thrombocyte reduction is not common, and platelets may actually increase. The leukocyte count promptly returns toward normal levels in most cases, and as it begins to increase, sufficient Cytoxan is administered to maintain it near 2000 to 5000/cu. mm. This may be accomplished by two intravenous injections weekly, or by oral administration, or by a combination of both routes. An oral dosage of 50 to 200 mg. daily or an intravenous injection of 5 mg./Kg. twice weekly will usually suffice.

The platelet and leukocyte counts should be followed carefully, and the prior treatment history of patients carefully evaluated as delineated above.

Leukopenia as a guide to adequacy of dosage — The best objective measure for dosage seems to be the number of circulating white blood cells. This is used as an index of the activity of the hematopoietic system, especially the bone marrow. The mechanism by which Cytoxan causes a reduction in the level of white blood cells is not known, but cessation of dosage results in an increase in the level, indicating that the hematopoietic system had not been permanently affected. When large doses (8 mg./Kg./day for 6 days) are given initially, the white cell count falls rapidly. Following the cessation of the 6-day course, the white cells may continue to decline for as long as 8 days and then increase. The reduction of the white cell count during Cytoxan therapy and its subsequent increase when therapy is discontinued can be repeated in the same patient. Maximal reduction in leukocyte count indicates the maximal permissible Cytoxan level for therapeutic effect. Leukopenic patients must be watched carefully for evidence of infection.

Total white blood cell and thrombocyte counts should be obtained 2 or more times weekly in order to evaluate therapy and to adjust dosage.

SIDE EFFECTS: Although Cytoxan is related to nitrogen mustard, it has no vesicant effect on tissue. It does not traumatize the vein when injected intravenously, nor does it cause any localized tissue reaction following extravasation. It may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally or directly into the tumor, when indicated. It is apparently active by each of these routes.

Nausea and vomiting are common and depend on dose and on individual susceptibility. However, many investigators accept the nausea and vomiting in favor of maintaining maximal therapy. The vomiting can be controlled with antiemetic agents.

Alopecia is a frequent side reaction to Cytoxan therapy. It has been observed in 28% of the patients studied in this country. The incidence is greater with larger doses. The loss of hair may first be noted about the 21st day of therapy and may proceed to alopecia totalis. This effect is reversed following discontinuance of Cytoxan; during reduced maintenance therapy, hair may reappear. It is essential to advise the patient in advance concerning this effect of the drug.

Dizziness of short duration and of minor degree has occasionally been reported.

Leukopenia is an expected effect and can be used as a guide to therapy. Thrombocytopenia may occur, especially after large doses. The leukocyte or platelet counts of an occasional patient may fall precipitously after even small doses of Cytoxan, as with all alkylating agents. The drug should be discontinued in such patients and reinstituted later at lower dosage after satisfactory hematologic recovery has occurred. Prior treatment with x-ray or with other chemotherapeutic agents frequently causes an earlier or exaggerated leukopenia or thrombocytopenia after Cytoxan medication. Only rarely has there been a report of erythrocyte or hemoglobin reduction.

ADMINISTRATION: Add 5 cc. sterile water (Water for Injection, U.S.P.) to 100 mg. of Cytoxan in the sterile vial (add 10 cc. to 200 mg. vial). Shake, allow to stand until clear, remove with sterile syringe and needle and inject.

The freshly prepared solution of Cytoxan may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally, or directly into the tumor. The solution should be administered promptly after being made but is satisfactory for use for three hours after preparation.

If the patient is receiving a parenteral infusion, the Cytoxan solution may be injected into the rubber tubing if the solution is glucose or saline.

No thrombosis or thrombophlebitis has been reported from injections of Cytoxan. Extravasation of the drug into the subcutaneous tissues does not result in local reactions.

PRECAUTIONS: Cytoxan should not be given to any person with a severe leukopenia, thrombocytopenia, or bone marrow infiltrated with malignant cells. It may be given with suitable precautions to patients who have had recent x-ray treatment, recent treatment with a cytotoxic agent, a surgical procedure within 2-3 weeks, or debilitated patients.

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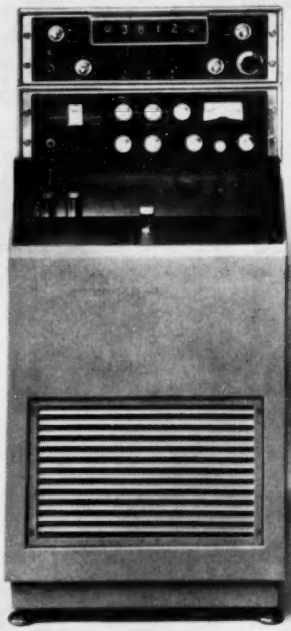

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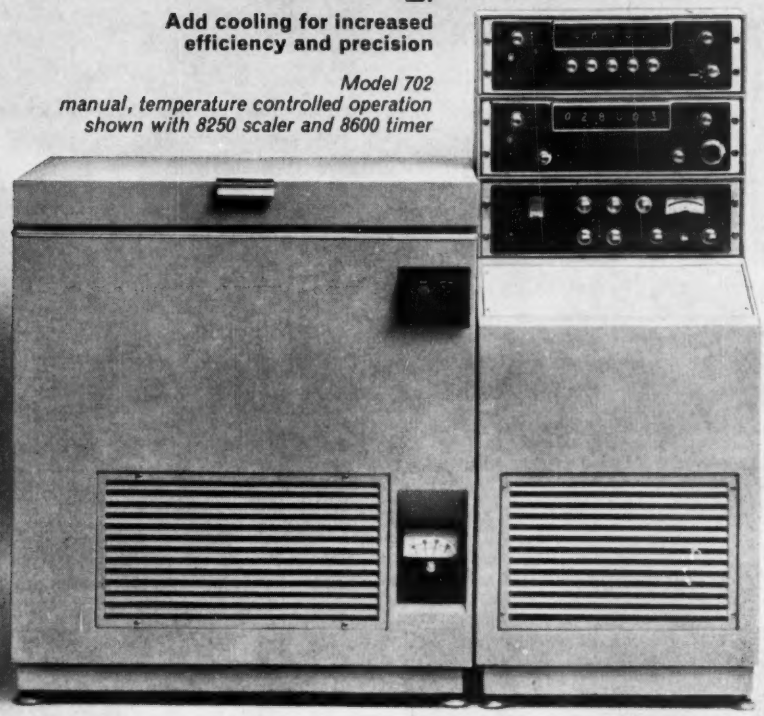

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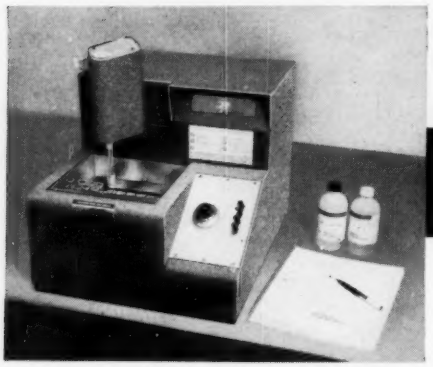

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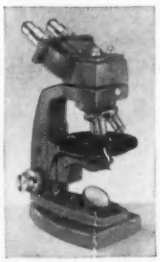

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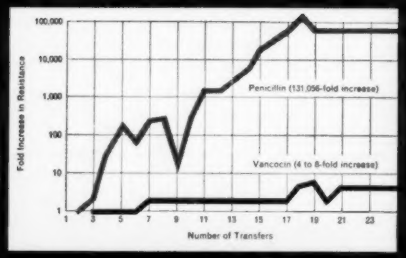

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THE QUANTITATIVE DETERMINATION OF IRON KINETICS AND HEMOGLOBIN SYNTHESIS IN HUMAN SUBJECTS *

By MYRON POLLYCOVE and ROBERT MORTIMER

(From the Donner Laboratory, Donner Pavilion, and the Lawrence Radiation Laboratory, University of California, Berkeley; and the Radioisotope Service and Medical Service, Veterans Administration Hospital, Boston, Mass.)

(Submitted for publication July 25, 1960; accepted January 26, 1961)

Two-thirds of the iron normally present in the body is in hemoglobin. Hemoglobin turnover is generally accepted to be much greater than turnover of iron in storage depots, in which nearly all of the remaining iron is found. For these reasons it was anticipated that hemoglobin synthesis might be measured directly by means of radioactive iron. The pioneering work of Huff and Elmlinger and their associates (1–3) proposed that hemoglobin synthesis could be determined from the product of the rate of removal of iron from plasma (plasma iron turnover, milligrams per day) and the fraction of radioactive iron incorporated into circulating erythrocytes. Further studies by Huff and others (4–16), however, revealed that hemoglobin synthesis calculated in this way was consistently greater than would be expected from relating circulating hemoglobin to well established data concerning the life span of erythrocytes. Plasma iron turnover data yielded daily hemoglobin synthesis rates of 1.2 to 2.0 per cent of circulating hemoglobin as contrasted with expected rates of 0.75 to 1.00 per cent corresponding to an erythrocyte life span range of 100 to 130 days (17–20).

Although plasma iron turnover can be used for relative comparisons of erythropoiesis under certain circumstances, it is apparent that plasma iron turnover must be analyzed into its component parts before it can be used for quantitative determination of hemoglobin synthesis. This study was undertaken in an attempt to quantitate hemoglobin synthesis. For this purpose, mathematical models were developed that are compatible with experimental data obtained from over 400 subjects. Sequential measurements of radioiron were made in plasma, red cells, and at the body surface (21, 22). Data obtained from 13 normal subjects and 6 patients with endogenous hemochromatosis are presented in this report and analyzed in accordance with the proposed models. Most patients with endogenous hemochromatosis are not anemic and show normal blood and marrow erythrons upon histologic examination, suggesting normal hemoglobin synthesis and erythrocyte life span (23). Iron kinetics in these patients is greatly altered by a marked pathologic increase of iron stores. Study of patients with endogenous hemochromatosis therefore provides an especially useful test of the proposed iron kinetics model.

MATERIALS AND METHODS

All normal subjects (1 female and 12 male volunteers between the ages of 24 and 72 years) studied with the methods presented are included in this report. All were asymptomatic, in good health, with normal hematologic values.

All patients with endogenous hemochromatosis studied (6 males) are included in this report. None had received blood transfusion or iron therapy. Five of these patients were between 50 and 60 years old, with characteristic slate-grey or leaden, dusky skin color, and diabetes. Diagnosis was established by the abnormally increased concentration of iron in plasma (178 to 278 μg per 100 ml), decreased total plasma iron-binding capacity (224 to 278 μg per 100 ml), and liver biopsy demonstrating characteristic periportal fibrosis in association with massive deposits of hemosiderin. The sixth patient was only 22 years old; although biopsy of his liver demonstrated markedly increased amounts of hemosiderin, no periportal fibrosis was present. Glucose tolerance and cardiac and hepatic function were normal. Plasma iron concentration (149 μg per 100 ml) and total binding capacity (314 μg per 100 ml) were normal; he had not been venesected. The diagnosis was established by demonstration of increased absorption of ingested iron [29 per cent of 4 mg iron (ferrous citrate) without ascorbic acid], in association with a striking familial incidence of endogenous hemochromatosis—his father and one paternal uncle had died with endogenous hemochromatosis and diabetes, and a living paternal uncle is also so affected. More precisely, since no pathologic organ function or morphology was demonstrable, he is classified as being at a prehemochromatotic stage of endogenous hemochromatosis (24).

* This investigation was supported in part by a grant from the United States Public Health Service (C-1440).

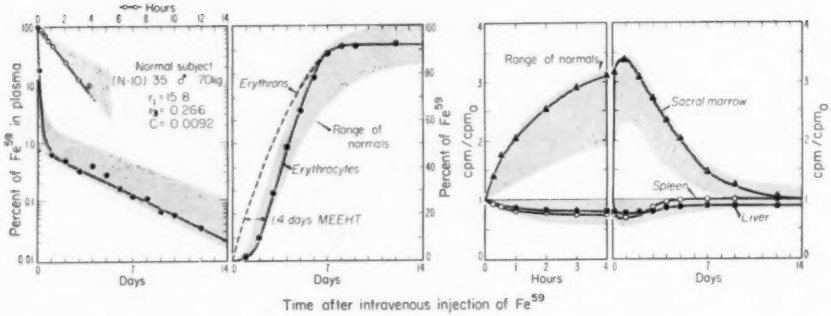

FIG. 1. RADIOIRON IN PLASMA AND ERYTHROCYTES, AND SURFACE RADIOACTIVITY IN A NORMAL SUBJECT. The interrupted curve, showing cumulative fixation of radioiron in erythrons, and the mean effective erythron hemoglobinization time (MEEHT) are calculated as shown in Appendices C and D.

Ten to 40 μc of radioactive iron (Fe^{59}), as ferrous citrate or ferric ammonium citrate, specific activity 1 to 15 μc per μg, was used. This was added to sufficient fresh plasma (the recipient's plasma is used except when it lacks sufficient latent iron-binding capacity) to ensure binding of all iron to the β_1-globulin, transferrin. After incubation at 37° C for 20 to 30 minutes, 5 to 20 ml of the labeled fresh plasma was injected intravenously with a calibrated syringe. Since iron in the plasma is entirely bound to transferrin, iron was injected in this form to ensure its acting as a true tracer. Others have given radioiron directly by i.v. injection and report that this mode of administration is satisfactory (10, 25). Perhaps radioiron may be used in this way as a tracer if given slowly so as not to exceed the plasma iron-binding capacity of the small mixing volume in transit from the site of injection to the lungs. During the 10 to 14 days following injection, serial blood samples were analyzed for plasma and erythrocyte radioactivity, and surface measurements of radioactivity over liver, spleen, and sacral bone marrow were made.

Plasma and erythrocyte radioactivity were measured by counting 2- or 3-ml samples in a well scintillation counter, with a 2-inch NaI crystal and 6-inch lead shielding (26). The counting efficiency was about 25 per cent for Fe^{59} and the background approximately 105 cpm. Blood samples were centrifuged at 2,000 G for 15 minutes to obtain plasma free of erythrocytes. It was necessary to take care in drawing and manipulating the blood to avoid hemolysis which, after significant labeling of erythrocytes occurs, would produce erroneously high concentration of radioiron in plasma (Figure 14, N–9). Concentration of iron in plasma and latent iron-binding capacity were determined by the modification of Peters and co-workers of the Ramsey method (27). Hematocrits were determined on all samples. Plasma and red cell volumes were determined independently by the Fe^{59} and P^{32} dilution methods (28, 29). Surface counting rates were obtained from mobile scintillation counters over liver and spleen, angled posteriorly from the anterior and midaxillary lines, respectively, and in the posterior midline over the broad upper portion of the sacrum as shown in Figures 21 and 22 in Appendix F (2, 3). A counting rate was also obtained over the anterior aspect of the thigh at a level 12 cm above the upper border of the patella. This position at the lower end of the femur is

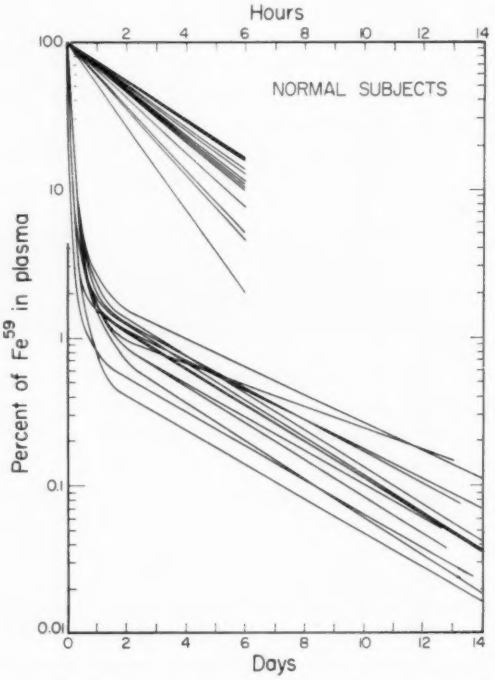

FIG. 2. PER CENT OF RADIOIRON IN PLASMA AFTER INTRAVENOUS INJECTION, SHOWING RAPID AND SLOW EXPONENTIAL COMPONENTS. Plasma radioiron data in 3 representative normal subjects are shown in Figure 14.

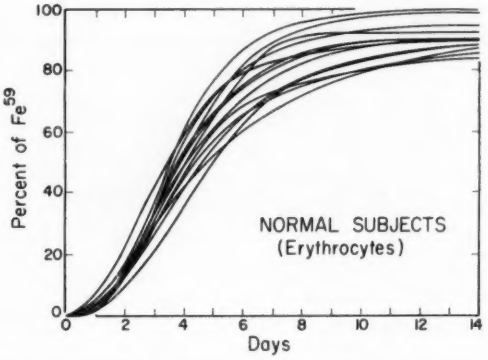

FIG. 3. PER CENT OF RADIOIRON IN CIRCULATING RED CELL MASS AFTER INTRAVENOUS INJECTION.

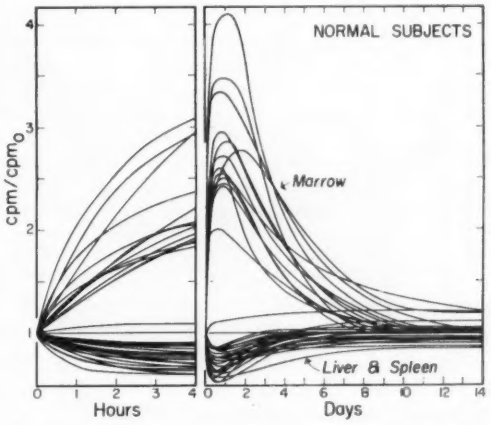

FIG. 4. RATIOS OF SACRAL, HEPATIC, AND SPLENIC SURFACE COUNTING RATES TO INITIAL RATE AFTER INTRAVENOUS INJECTION OF RADIOIRON.

used to detect increase of red marrow volume in adults and to check variation of background and instrumentation.

MATHEMATICAL ANALYSIS OF PLASMA RADIOIRON

Study of normal subjects for whom tracer radioiron was measured in plasma, sacral marrow, liver, spleen, and circulating erythrocytes (Figures 1–4), results in the following observations. Virtually all radioactive iron moves rapidly from plasma to bone marrow, remaining there longer than 1 day. During the next 4 to 6 days, radioiron leaves the marrow and simultaneously appears within circulating erythrocytes. In plasma, during the first 5 to 8 hours, there is a rapid exponential decrease of radioiron, the rate of which subsequently diminishes. After approximately 2 days, a second, slower exponential rate of decrease of radioiron is established. This persists for 8 to 12 days (Figures 1, 2). The presence of this second component is evidence for feedback of radioactive iron into plasma from another iron pool. Since virtually all plasma radioiron transfers to marrow, it seems plausible that the feedback occurs from marrow. It can be shown that, if most of the radioiron feedback were to occur from a labile storage pool rather

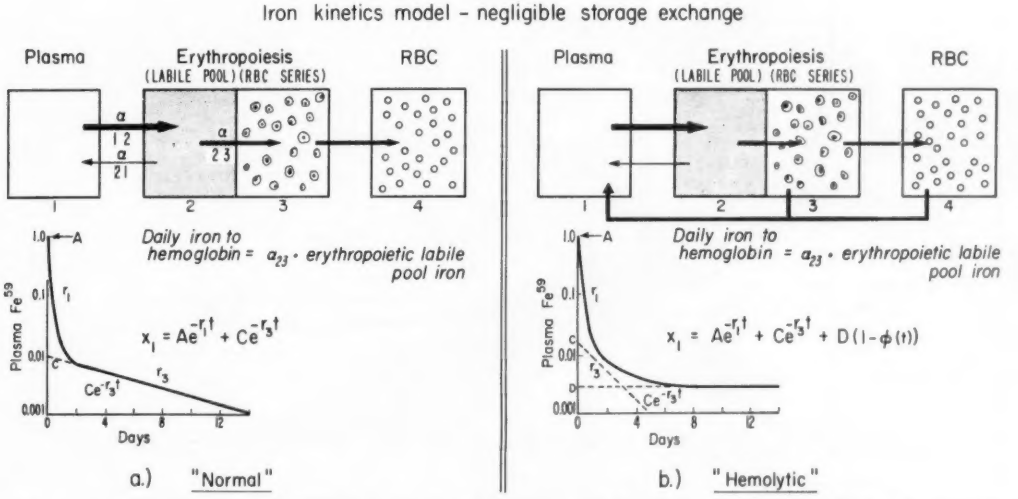

FIG. 5. IRON KINETICS MODEL PROPOSED TO APPLY UNDER CONDITIONS OF NEGLIGIBLE PLASMA-STORAGE IRON EXCHANGE AND SOLUTION OF THE CORRESPONDING DIFFERENTIAL EQUATIONS. In the absence of hemolysis, plasma radioiron continues to decrease exponentially for 2 weeks. Hemolysis of maturing erythrons or circulating erythrocytes results in feedback of radioiron to plasma and early equilibration of plasma radioiron at a constant level.

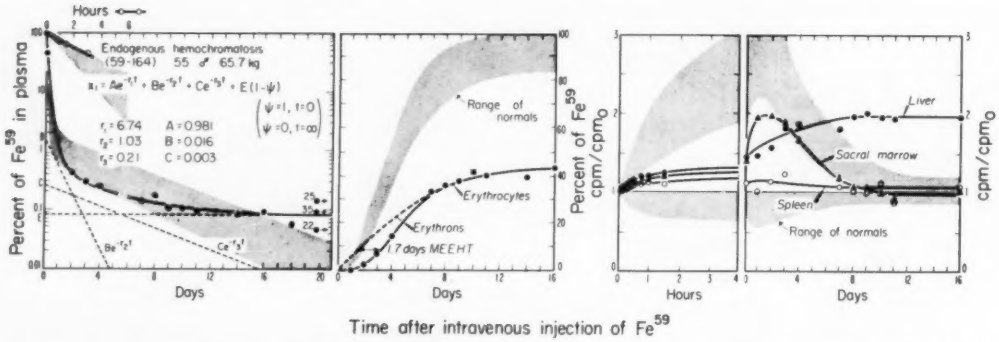

FIG. 6. RADIOIRON IN PLASMA AND ERYTHROCYTES, AND SURFACE RADIOACTIVITY IN A PATIENT WITH ENDOGENOUS HEMOCHROMATOSIS. The interrupted curve, showing cumulative fixation of radioiron in erythrons, and the MEEHT are calculated as shown in Appendices C and D.

than a labile erythropoietic pool, most of the radioiron would transfer initially into the former instead of the latter pool and, since almost half of storage iron is in liver (30), would thereby markedly increase the hepatic counting rate. Reappearance in plasma of measurable radioactive iron from destroyed circulating erythrocytes and storage depots is normally delayed beyond the 10 to 14 day period of measurement.

From these considerations the model in Figure 5a is proposed for normal subjects whenever storage deposition is a very small fraction of plasma iron exchange. Solution of the corresponding simultaneous differential equations is presented in Appendix A. The plasma radioiron can be expressed as a function of time by the equation $x_1 = Ae^{-r_1 t} + Ce^{-r_3 t}$. The slope r_1 is determined largely by the rate of transfer of radioiron from plasma into the labile erythropoietic iron pool (fraction of plasma iron transferred daily); the slope r_3 is determined largely by the rate of transfer of radioiron from the labile erythropoietic iron pool into maturing erythrons (fraction of

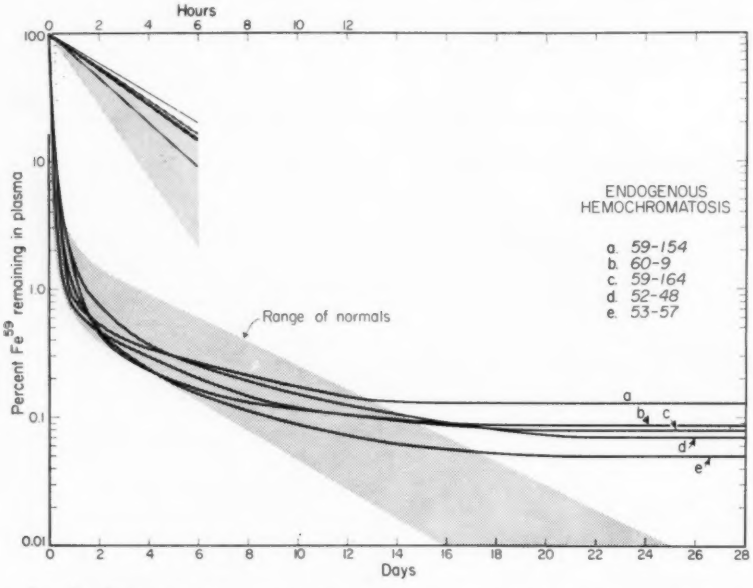

FIG. 7. PER CENT OF RADIOIRON IN PLASMA AFTER INTRAVENOUS INJECTION, SHOWING INITIAL RAPID EXPONENTIAL COMPONENT AND FINAL CONSTANT EQUILIBRIUM LEVEL. Graphic analysis reveals two additional exponential components (Figure 10). Plasma radioiron data in 3 representative patients with endogenous hemochromatosis are shown in Figure 10.

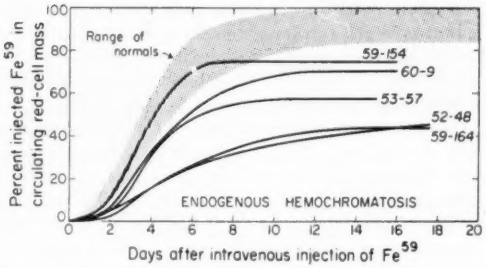

FIG. 8. PER CENT OF RADIOIRON IN CIRCULATING RED CELL MASS AFTER INTRAVENOUS INJECTION.

erythropoietic pool transferred daily). Measurement of the two plasma radioiron slopes and their intercepts, together with use of the formulae summarized in Appendix D, permits calculation of the intercompartmental rate constants and the amount of iron in the labile erythropoietic pool. Calculation of the rates of irreversible iron fixation in maturing erythrons and the corresponding rates of hemoglobin synthesis is shown in Appendix B.

When considerable storage deposition of plasma iron occurs (as in patients with hemochromatosis, hemosiderosis, or decreased erythropoiesis) the model shown in Figure 5 is not adequate. Under these circumstances, initial hepatic deposition of radioiron occurs along with transfer to marrow and erythrons, and there is a corresponding decrease in the percentage of radioiron incorporated into circulating erythrocytes (Figures 6, 8, 9). This indicates that deposition of iron in relatively fixed stores occurs simultaneously with incorporation of iron from labile marrow pool into maturing erythrons (Figure 6). Graphic analysis of the plasma radioiron curve reveals distinctly more than two exponential components (Figure 10) which may be arbitrarily resolved into three exponential components. This pattern suggests feedback of radioiron from at least two distinct iron pools into the plasma pool. Under these circumstances, it is postulated that plasma iron exchanges with a storage pool in addition to an erythropoietic pool.

From these considerations the generally applicable

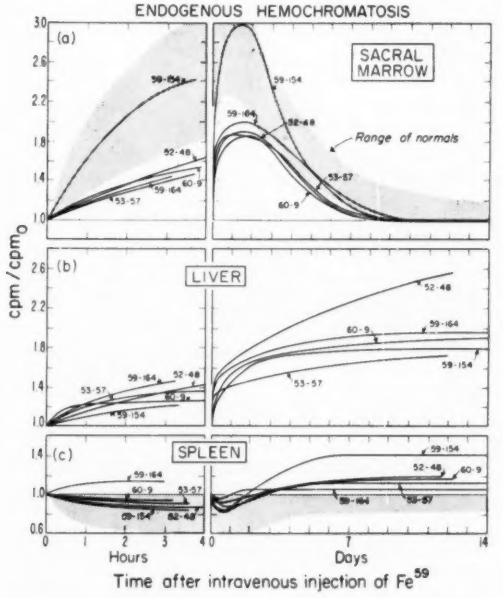

FIG. 9. RATIOS OF SACRAL, HEPATIC, AND SPLENIC SURFACE COUNTING RATES TO INITIAL RATE AFTER INTRAVENOUS INJECTION OF RADIOIRON.

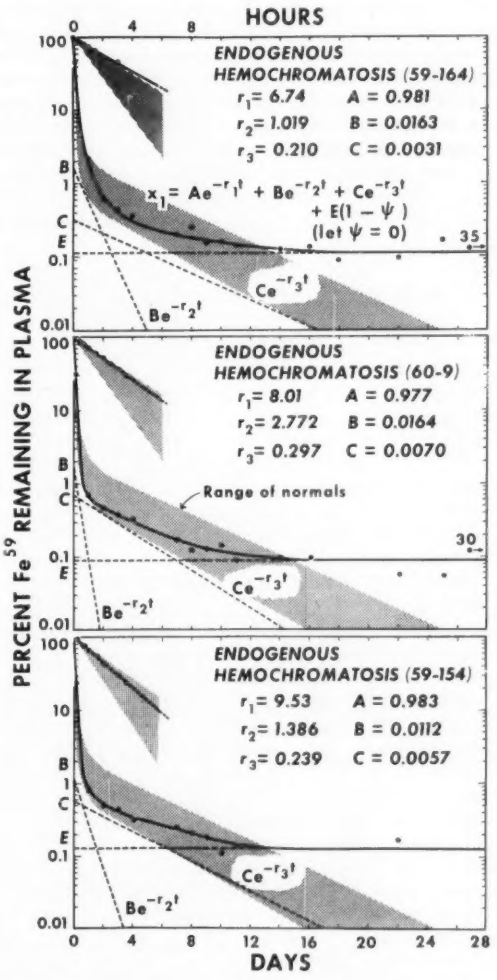
HOURS
ENDOGENOUS HEMOCHROMATOSIS (59-164)
r_1= 6.74 A = 0.981
r_2= 1.019 B = 0.0163
r_3= 0.210 C = 0.0031
$x_1 = Ae^{-r_1 t} + Be^{-r_2 t} + Ce^{-r_3 t} + E(1 - \psi)$
(let $\psi = 0$)
ENDOGENOUS HEMOCHROMATOSIS (60-9)
r_1= 8.01 A = 0.977
r_2= 2.772 B = 0.0164
r_3= 0.297 C = 0.0070
Range of normals
ENDOGENOUS HEMOCHROMATOSIS (59-154)
r_1= 9.53 A = 0.983
r_2= 1.386 B = 0.0112
r_3= 0.239 C = 0.0057
PERCENT Fe^{59} REMAINING IN PLASMA
DAYS

FIG. 10. PER CENT OF RADIOIRON IN PLASMA AFTER INTRAVENOUS INJECTION. Plasma radioiron data in 3 representative patients with endogenous hemochromatosis are shown.

model shown in Figure 11 is proposed. Neglecting the slow return to plasma of iron from storage reserve, solution of the corresponding simultaneous differential equations is presented in Appendix A. The plasma radioiron can be expressed as the sum of three exponential components, $x_1 = Ae^{-r_1 t} + Be^{-r_2 t} + Ce^{-r_3 t}$. The magnitude of the slopes r_1 and r_2 corresponds to those observed for normal subjects. The additional slope observed when appreciable storage deposition of plasma iron occurs, r_3, is largely determined by the rate of transfer of radioiron from the labile storage pool into relatively fixed iron stores (fraction of storage pool transferred daily); it is considerably smaller than r_1 and larger than r_2. Slope r_3 is determined by irreversible transfer of iron from the labile erythropoietic pool into erythrons. Measurement of the three plasma radioiron slopes, their intercepts, and f, the maximal fraction of radioiron incorporated into erythrons, together with the use of the formulae summarized in Appendix D, permits calculation of the amounts of iron in the labile erythropoietic and labile storage pools. Calculation of the rates of iron deposition in stores, irreversible fixation of iron in maturing erythrons, and corresponding hemoglobin synthesis is shown in Appendix B. The simple model is seen to be a special case of the more general storage model when $\alpha_{13} = 0$.

The rate of hemoglobin synthesis can be calculated from the daily amount of iron fixed irreversibly in maturing erythrons, using the relation: 1 g hemoglobin contains 3.4 mg iron. This involves the assumption that the iron present in mature erythrocytes is almost entirely in hemoglobin. The calculation of hemoglobin synthesis involves measurement of the initial slope, r_1, and total plasma iron, X_1, measured during the initial 4 hour period of study. The assumption of steady state conditions implies that the initial plasma iron turnover, $\alpha_{12}X_1$, is equal to the average turnover during the 2 week period of study. The concentration of iron in plasma of normal subjects varies daily, being approximately 15 per cent less at the end of the day before sleep than at the beginning of day after rising (31, 32). Plasma iron turnover, however, is probably more constant, since this is almost entirely determined by the number of erythrons and storage iron present. Plasma iron turnover in normal subjects is relatively constant throughout the wide range

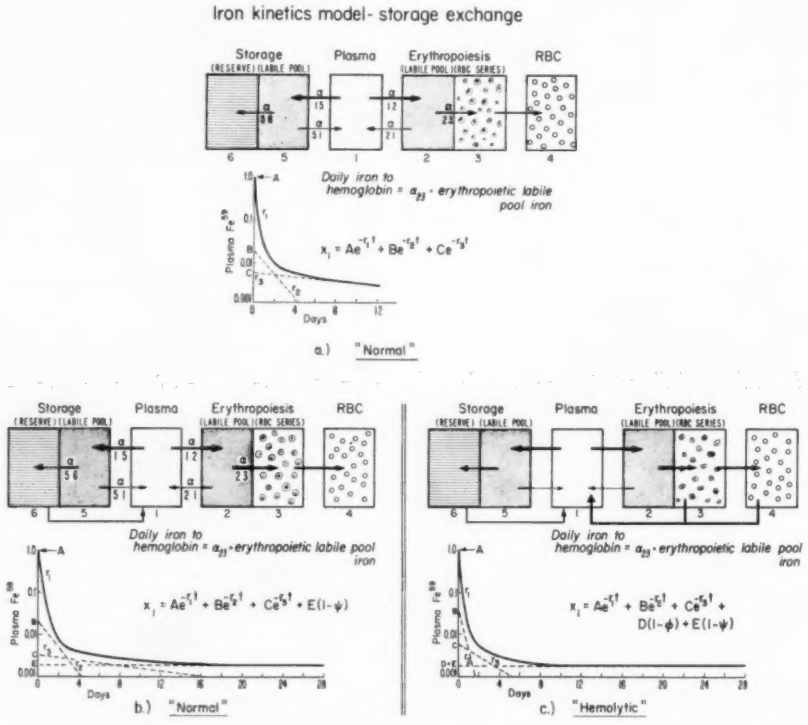
Iron kinetics model- storage exchange
Storage
(RESERVE) (LABILE POOL)
Plasma
Erythropoiesis
(LABILE POOL) (RBC SERIES)
RBC
Daily iron to hemoglobin = α_{23} · erythropoietic labile pool iron
$x_1 = Ae^{-r_1 t} + Be^{-r_2 t} + Ce^{-r_3 t}$
Plasma Fe^{59}
Days
a.) "Normal"
$x_1 = Ae^{-r_1 t} + Be^{-r_2 t} + Ce^{-r_3 t} + E(1-\psi)$
b.) "Normal"
$x_1 = Ae^{-r_1 t} + Be^{-r_2 t} + Ce^{-r_3 t} + D(1-\phi) + E(1-\psi)$
c.) "Hemolytic"

FIG. 11. IRON KINETICS MODEL PROPOSED TO INCLUDE PLASMA-STORAGE IRON EXCHANGE AND SOLUTION OF THE CORRESPONDING DIFFERENTIAL EQUATIONS. In the absence of hemolysis, plasma radioiron slowly equilibrates at a constant level after 2 weeks as a result of slow feedback of radioiron to plasma from the miscible reserve storage iron pool. Hemolysis of maturing erythrons or circulating erythrocytes results in more rapid feedback of radioiron to plasma and early equilibration of plasma radioiron at a constant level.

of plasma iron concentration of 70 to 170 μg per 100 ml, the fraction of plasma iron removed per hour increasing as total plasma iron decreases, thereby maintaining a constant product.

The mean life span of the erythron is estimated by the ratio of total hemoglobin to daily hemoglobin breakdown. The latter is calculated from the rate of hemoglobin synthesis and the change, if any, in total hemoglobin (Appendix B). The calculation of mean erythron life span involves the assumption that the average rate of hemoglobin breakdown, calculated from measurements during a 2 week period, remains constant during the life span of the erythron. *Mean erythron life span* in this context signifies the life span corresponding to the calculated average rate of hemoglobin breakdown. The mean time interval between incorporation of iron into marrow erythrons and its appearance in circulating erythrocytes is designated as the *mean effective erythron hemoglobinization time* (MEEHT). The method of calculating cumulative net incorporation of radioiron into maturing erythrons and MEEHT is presented in Appendix C. The simpler mathematical model (Figure 5) in which plasma iron exchange with storage pools is neglected can be applied, not only to normal subjects, but to a variety of conditions in which the normal pattern of iron flow from plasma to marrow to erythrocytes occurs. This includes iron deficiency anemia, primary or secondary polycythemia, and most hemolytic anemias (21, 22, 33, 34). Premature re-entry of radioiron into plasma results whenever erythron hemolysis occurs, whether in marrow (associated with increased MEEHT) or subsequent to erythron entry into the circulation [frequently associated with *delayed* increase of splenic counting rate above its initial level; i.e., cpm per $cpm_0 > 1$ (22)]. In hemolytic anemias, feedback of radioiron from destroyed labeled erythrocytes to plasma results in a progressive decrease of the negative plasma radioiron slope (which may even become positive in severe hemolysis), terminating in a constant equilibrium level (22). Under these circumstances, the exponential components may be approximated by subtraction of the minimal value from the preceding curve.

This mathematical treatment of hemolytic feedback is a simple approximation that is very useful and yields results that agree closely with more complex and precise methods. The approach is not rigorous, since it is apparent that feedback of radioiron from hemolyzed erythrons to plasma is not present at a constant level from time of injection through the entire 2 week period of study. Detailed analysis of this problem, with evaluation of the hemolytic component will be presented separately, together with results obtained in studies of patients with hemolytic anemias (21). The term $D[1 - \phi(t)]$, $\phi(t) = 1$ at $t = 0$ and $\phi(t) = 0$ at $t = \infty$ (Figures 5, 11) represents the component of plasma radioiron resulting from hemolysis. A simple approximation is obtained by setting $\phi(t) = 0$, D being the measured final constant value of plasma radioiron. This simplification alters the calculated rate of hemoglobin synthesis and derived mean erythron life span by less than 10 per cent, as compared with values derived from use of $D[1 - \phi(t)]$.

Feedback of radioiron from relatively fixed stores to plasma becomes appreciable in 14 to 21 days. Considerations similar to those discussed for hemolytic feedback apply, and $x_1 = Ae^{-r_1 t} + Be^{-r_2 t} + Ce^{-r_3 t} + E[1 - \psi(t)]$ (Figure 11b). Since the final "constant" plasma radioiron level E, resulting from storage feedback, usually occurs at a later time and a lower level than that resulting from hemolytic feedback, a smaller error (less than 5 per cent) is introduced by subtracting this constant minimal value from the preceding curve. A more rigorous and complex mathematical treatment of feedback from relatively fixed stores (compartment 6 illustrated in Figure 6) will be presented separately. The amount of storage iron that is miscible in approximately 3 weeks is calculated by relating the "constant" plasma radioiron level E to the fraction of injected radioiron deposited in storage, $1 - f$, as shown in Appendix B. In the absence of hemolysis, radioiron continues to be slowly removed from storage reserve and is incorporated via plasma into circulating erythrocytes. The so-called constant plasma level E is, in fact, a slowly decreasing one, but with too shallow a slope to be evident during the 4 to 5 week period of observations.

All radioiron is initially in plasma, and 7 to 10 days later is normally almost entirely in circulating erythrocytes. Initial surface radioactivity rates over liver, spleen, and marrow, cpm_0, obtained by extrapolation back to $t = 0$, are largely the result of radioactivity in plasma circulating through these organs. As radioiron leaves plasma and accumulates in marrow, splenic and hepatic counting rates usually fall, while the sacral counting rate rises rapidly. Subsequently, *in vivo* counting rates normally return to their initial value, cpm_0, when almost all radioiron has returned to the blood (Figures 1, 4). For convenience, body surface radioactivity is *not* expressed as *net* radioactivity, $cpm - cpm_0 \times$ (fraction of radioiron in circulating blood), but rather as the ratio cpm/cpm_0. Expressed in this way, hepatic and splenic radioactivity normally do not exceed 1, sacral radioactivity usually exceeds 2 within 4 hours, and all sites approximate 1 within 10 days (Figure 4). Slow increase of surface hepatic radioactivity during the first 3 hours after injection, together with simultaneous slow disappearance of radioiron from plasma (small r_1), is almost always the result of considerable iron exchange between plasma and stores (Figures 6, 7, 9); while relatively rapid initial hepatic accumulation of radioiron, comparable with the rate of normal sacral accumulation of radioiron, is associated with hemoglobin synthesis in hepatic erythrons and subsequent decrease of hepatic radioactivity (2, 21, 22). Slow initial increase of hepatic radioactivity (not present in patients with low concentration of iron in plasma) with confirmatory presence of a distinct third exponential component in the plasma radioiron curve, and subnormal incorporation of radioiron into circulating erythrocytes, indicates application of the storage ex-

change model (Figure 11). Unless there is gradual *initial* increase of hepatic radioactivity that persists (Figure 9), the "normal flow" model is used (Figure 5). In so neglecting normal storage iron deposition, not more than a 10 per cent error is incurred in calculating hemoglobin synthesis and mean erythron life span, as shown in the Discussion. Because of the experimental error (± 5 per cent) involved in red cell volume determinations used to determine f, it does not seem advantageous to use the storage exchange model for more exact measurement of normal deposition of stored plasma iron.

The storage exchange model involves the variable f, the maximal fraction of injected radioiron incorporated into erythrons. When hemolysis is absent, as is the case in 5 of the patients with hemochromatosis, f equals the measured maximal incorporation of radioiron into circulating erythrocytes (Figures 6 and 8). If erythrons are destroyed within the 10 day period after incorporation of radioiron, then f is greater than maximal *net* incorporation of radioiron into circulating erythrocytes. The occurrence of hemolysis usually is evidenced by cessation of the decrease in plasma radioiron and establishment of a constant equilibrium level in 10 days or less (21, 22, 34). Plasma radioiron would not be expected to reach a constant level in less than 10 days whenever hemolysis is solely the result of a shortened finite erythrocyte life span longer than 10 days. Finite erythrocyte life span is here defined as the occurrence of erythrocyte destruction with normal distribution about a definite time interval after erythron release into the circulation. Occurrence of only this type of hemolysis is probably rare, since complete study of over 400 patients with shortened erythrocyte life span has demonstrated equilibration of plasma radioiron at a constant level within 10 days (21).

This early stabilization of plasma radioiron is present in this study only in hemochromatotic Patient 59–70 with mild hemolysis resulting in a mean erythron life span of 52 days; plasma radioiron in this patient attains a constant level by Day 9 (Figure 12). In this case f is greater than maximal erythrocyte *net* incorporation of radioiron (Figure 12). To arrive at an estimate of f when significant hemolysis and plasma-storage iron exchange are both present, surface measurements may be used as shown in Appendix E. Measurements of radioiron in liver, spleen, marrow, blood, and occasionally lung, are used in an effort to account in rough approximation for the distribution of radioiron in the body throughout the study. With rare exceptions it has been possible to do this in over 400 studies, including cases in which there is loss of radioiron by gastrointestinal bleeding or pulmonary bleeding (21, 28). These semiquantitative estimates derived from surface counting allow better localization and quantitation of pathologic function and are helpful in selecting the appropriate model and value for f used for quantitative analysis of plasma and erythrocyte radioiron data (Appendix E). Most patients with appreciable hemolysis, however, do not have appreciable plasma-storage iron exchange relative to plasma-erythropoiesis iron exchange, and the "normal flow" model is used—i.e., $f = 1$.

When the storage exchange model involving the sum of three exponentials is used, the slopes and intercepts obtained from the plasma radioiron curves are quite sensitive to the curve-fitting employed. Although the values for compartment size and transfer rate are very sensitive to errors in the graphically determined slopes and intercepts, these changes are compensatory with respect to the *amounts* of iron transferred. For example, if at a given level of plasma radioiron, r_3 becomes greater, C also becomes larger, the calculated erythropoietic labile pool (X_2) smaller, and the rate constants α_{22} and α_{21} become larger; the products $\alpha_{22}X_2$ (iron fixed for hemoglobin synthesis) and $\alpha_{21}X_2$ (iron feedback to plasma), however, remain relatively constant. Measurement of the rate of appearance of radioiron in circulating erythrocytes is very useful as an independent rough check of the applicability of the calculated transfer rate from the erythropoietic labile pool compartment (X_2) to the fixed erythron iron compartment (X_3). This transfer rate into erythrons must be more rapid than the emergence

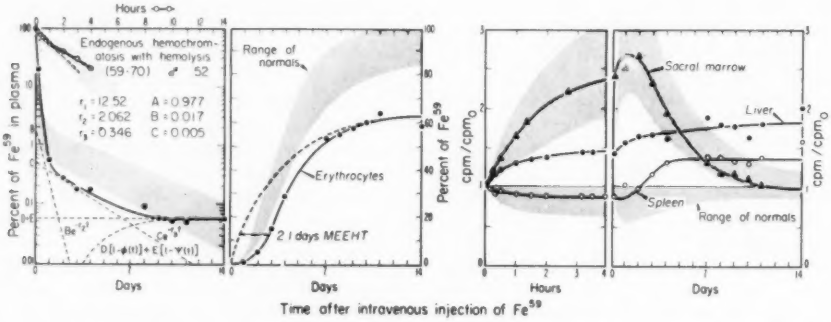

FIG. 12. RADIOIRON IN PLASMA AND ERYTHROCYTES, AND SURFACE RADIOACTIVITY IN A PATIENT WITH ENDOGENOUS HEMOCHROMATOSIS AND MILD HEMOLYSIS. Plasma radioiron stabilizes at a constant level within 9 days as a result of the hemolytic component $D[1 - \phi(t)]$ superimposed upon the more gradual storage feedback component $E[1 - \psi(t)]$.

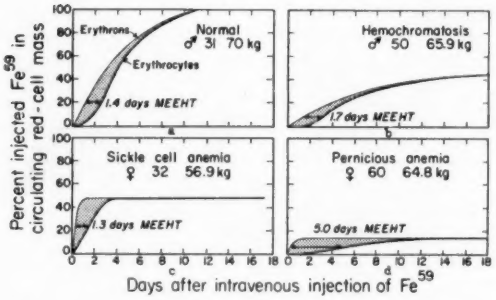

FIG. 13. CUMULATIVE NET INCORPORATION OF RADIOIRON INTO ERYTHRONS AND CIRCULATING ERYTHROCYTES AND MEEHT. See Appendix C.

of radioiron in circulating erythrocytes (Figures 1, 6, 12, 13). Because of random errors in plasma data obtained at very low levels of radioactivity and errors involved in determining f, $D(1-\phi)$, and $E(1-\psi)$, analysis of the plasma curve must be integrated with measurements of red cell, marrow, liver, and spleen radioiron to obtain the most accurate and meaningful results.

RESULTS

Results obtained in 13 normal subjects are shown in Table I. These include experimental data obtained by direct measurements and calculated intercompartmental rate constants, labile erythropoietic iron pool size, daily hemoglobin synthesis, mean erythrocyte life span, and mean effective erythron hemoglobinization time. The plasma radioiron curves of this group can be resolved into two exponential components (Figures 1, 2, 14). Using these slopes and their intercepts, the following average estimates are obtained in normal subjects: 32.5 mg of iron leaves plasma daily ($\alpha_{12}X_1$). Of this, 21.4 mg ($\alpha_{23}X_2$)—i.e., approximately two-thirds, or $\alpha_{23}/(\alpha_{23}+\alpha_{21})$—is used for daily hemoglobin synthesis. Expressed in another way, the daily feedback of iron, 11.1 mg ($\alpha_{21}X_2$), from the labile erythropoietic pool (X_2), mean value 84.9 mg, to plasma pool (X_1), mean value 3.6 mg, is approximately one-third, $\alpha_{21}/(\alpha_{23}+\alpha_{21})$, of the amount of iron, 32.5 mg, daily entering the labile erythropoietic pool from plasma. Normal daily hemoglobin synthesis is 6.3 ± 0.9 (1 SD) g per day (55 to 84 kg, weight range) or 1.3 ± 0.2 g per L blood per day. Assuming a steady state, verified during the course of each individual studied, we calculate a mean erythrocyte life span of 117 ± 7.5 days. The esti-

TABLE I

Data derived from normal subjects

Subject	Age, Sex, Wt	Hb	Hct	MCHC*	RCV†	Total body Hb	$t_{\frac{1}{2}}$	SI	BV	PV	Total plasma iron X_1	First plasma Fe^{59} slope r_1	Second plasma Fe^{59} slope r_2
	yrs *kg*	*g/100 ml*	*%*	*%*	*ml*	*g*	*hrs*	*μg/100 ml*	*ml*	*ml*	mg		
N- 1	62 ♀ 75.0	14.0	42.4	0.33	1,540	509	2.25	101	4,140	2,600	2.6	7.4	0.289
N- 2	35 ♂ 78.0	15.9	42.5	0.37	2,360	883	1.80	112	5,590	3,230	3.3	9.3	0.266
N- 3	28 ♂ 66.8	15.4	44.0	0.35	2,090	731	1.83	144	4,750	2,660	4.2	9.1	0.289
N- 4	32 ♂ 70.0	17.1	49.1	0.35	2,130	745	2.32	200	4,490	2,360	4.7	7.2	0.289
N- 5	29 ♂ 84.0	14.5	44.0	0.33	2,180	928	1.60	117	5,780	2,970	3.5	10.4	0.306
N- 6	26 ♂ 75.0	15.2	44.9	0.34	2,180	741	2.28	150	5,000	2,820	4.2	7.3	0.231
N- 7	26 ♂ 77.0	14.0	45.0	0.31	2,410	749	1.89	119	5,360	2,950	3.8	9.0	0.238
N- 8	32 ♂ 68.2	15.0	46.9	0.32	2,010	643	2.00	130	4,460	2,450	3.2	8.3	0.210
N -9	72 ♂ 75.0	17.5	50.0	0.35	2,070	725	2.10	185	4,340	2,270	4.2	7.9	0.239
N-10	35 ♂ 69.5	14.8	45.0	0.33	2,280	750	1.05	74	5,550	3,220	2.4	15.8	0.266
N-11	24 ♂ 63.5	16.2	48.0	0.34	2,310	785	1.62	160	4,940	2,630	4.2	10.3	0.167
N-12	29 ♂ 72.7	14.1	44.6	0.32	2,350	751	1.35	86	5,730	3,380	2.9	12.3	0.289
N-13	33 ♂ 54.8	14.0	43.4	0.32	1,980	634	2.31	136	4,980	3,000	4.1	7.2	0.217
	Mean 71.5	15.2	45.4	0.33	2,150	738	1.88	132	5,010	2,810	3.6	9.3	0.254
	SD					±99	±0.38	±35			±0.7	±2.4	±0.039

* Mean corpuscular hemoglobin concentration.
† Red cell volume.

TABLE I—(*Continued*)

	Second plasma slope intercept C	Intercompartmental rate constants			Fe leaving plasma/day $\alpha_{12}X_1$	Erythropoietic labile iron pool X_2	Daily Fe for Hb synthesis $\alpha_{32}X_2$	Daily hemoglobin synthesis		MELS†	MEEHT§
		α_{12}	α_{22}	α_{21}							
Subject					*mg*	*mg*	*mg*	*g*	*g/L*	*days*	*days*
N- 1	0.0220	7.30	0.295	0.094	19.2	49.5	14.5	4.3	1.0	108	1.5
N- 2	0.0070	9.19	0.268	0.061	30.3	92.1	24.7	7.3	1.3	121	1.4
N- 3	0.0200	8.91	0.295	0.170	37.4	80.4	23.8	7.0	1.5	105	1.4
N- 4	0.0244	7.02	0.296	0.161	33.2	72.6	21.5	6.3	1.4	118	1.6
N- 5	0.0120	10.28	0.309	0.118	36.0	83.0	25.6	7.5	1.3	124	1.1
N- 6	0.0180	7.20	0.235	0.105	30.2	88.9	20.9	6.2	1.2	120	1.4
N- 7	0.0200	8.83	0.242	0.171	33.5	81.2	19.7	5.8	1.1	129	1.3
N- 8	0.0110	8.24	0.222	0.077	26.2	86.1	19.1	5.6	1.3	115	1.4
N- 9	0.0180	7.79	0.244	0.133	32.7	86.7	21.2	6.2	1.4	117	1.3
N-10	0.0092	15.66	0.268	0.141	37.4	91.4	24.5	7.2	1.4	104	1.6
N-11	0.0130	10.17	0.169	0.130	42.7	143.0	24.2	7.1	1.4	111	1.1
N-12	0.0153	12.11	0.293	0.180	35.1	74.2	21.8	6.4	1.1	117	1.6
N-13	0.0240	7.04	0.222	0.163	28.8	74.9	16.6	4.9	1.0	129	1.7
Mean	0.0165	9.21	0.258	0.131	32.5	84.9	21.4	6.3	1.3	117	1.4
SD	±0.0054	±2.36	±0.039	±0.037	±5.7	±19.9	±3.2	±0.9	±0.2	±7.5	±0.2

‡ Mean erythrocyte life span.
§ Mean effective erythron hemoglobinization time.

mated MEEHT is 1.4 ± 0.2 days. This quantity is a measure of the time interval between incorporation of iron into red cell precursors and its release into the vascular system.

Results obtained from six patients with endogenous hemochromatosis are shown in Table II. These include directly measured experimental data and calculated intercompartmental rate constants, labile erythropoietic and storage iron pools, daily iron storage deposition, miscible storage iron,

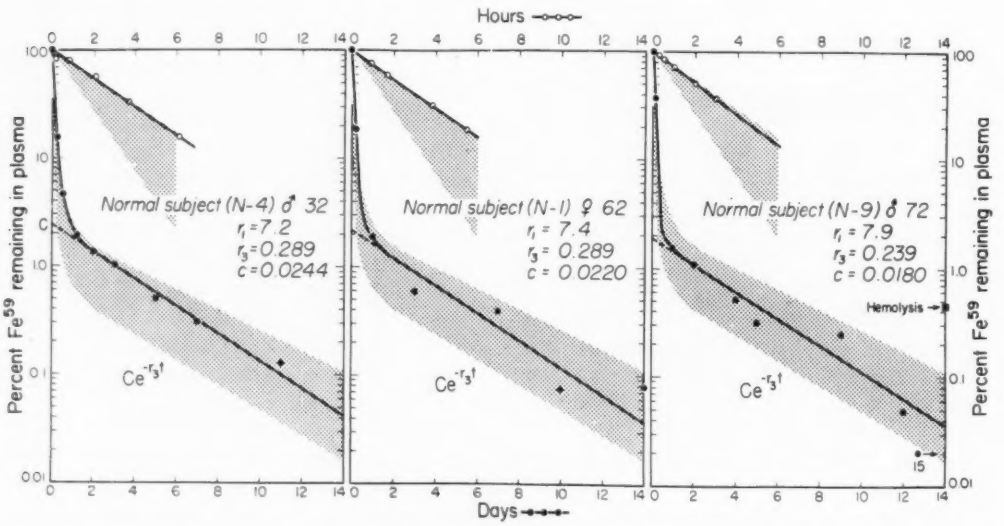

FIG. 14. PER CENT OF RADIOIRON IN PLASMA AFTER INTRAVENOUS INJECTION. Plasma radioiron data in 3 representative normal subjects are shown.

TABLE II

Data from six patients with endogenous hemochromatosis *

Subject	Additional diagnosis or remarks	Age, Sex, Wt	Hb	Hct	MCHC	RCV	Total body Hb	$t_{\frac{1}{2}}$	SI	BV	PV	Total plasma iron X_1	First plasma slope r_1	Second plasma slope r_2	Third plasma slope r_3	First intercept A	Second intercept B	Third intercept C
		yrs *kg*	*g/100 ml*	%	%	*ml*	*g*	*hrs*	*μg/100 ml*	*ml*	*ml*	*mg*						
53–57		59 ♂ 54.8	15.0	42.0	36	1,960	699	2.42	265	4,660	2,700	7.2	6.89	1.066	0.257	0.983	0.0120	0.0050
52–48		50 ♂ 65.9	14.4	46.7	31	1,730	540	2.27	248	4,270	2,540	6.3	7.34	1.155	0.204	0.979	0.0152	0.0050
59–154	Very early	22† ♂ 68.2	13.9	40.9	34	2,150	733	1.75	149	5,010	2,860	4.3	9.53	1.386	0.239	0.983	0.0112	0.0057
60–9	Study done 10 days $\bar{p}$ venesection	60 ♂ 64.1	13.3	40.5	33	2,300	759	2.08	178	7,210	4,910	8.8	8.01	2.772	0.297	0.977	0.0164	0.0070
59–164		55 ♂ 65.7	13.5	40.3	34	1,950	665	2.47	245	5,440	3,490	8.6	6.74	1.034	0.210	0.981	0.0160	0.0028
	Mean	56 63.7	14.0	42.1	34	2,020	679	2.20	217	5,320	3,300	7.0	7.70	1.483	0.241	0.981	0.0142	0.0053
	SD											±1.65						
59–70†		52 ♂ 66.8	13.4	38.9	35	2,170	748	1.33	197	5,620	3,450	6.8	12.52	2.062	0.346	0.977	0.0174	0.0052

TABLE II—(*Continued*)

Subject	Additional diagnosis or remarks	Age, Sex, Wt	f	Intercompartmental rate constants						Fe leaving plasma/day $(\alpha_{12}+\alpha_{13})X_1$	Erythropoietic labile iron pool X_2	Storage labile iron pool X_3	Miscible storage iron X_{ms}	Daily Fe for storage deposition $\alpha_{56}X_5$	Daily Fe for Hb synthesis $\alpha_{23}X_2$	Daily Hb synthesis	Daily Hb synthesis	MELS	MEEHT
				α_{12}	α_{23}	α_{21}	α_{13}	α_{56}	α_{51}										
		yrs *kg*								*mg*	*mg*	*mg*	*g*	*mg*	*mg*	*g*	*g/L*	*days*	*days*
53–57		59 ♂ 54.8	0.57	3.92	0.241	0.046	2.86	0.987	0.152	48.6	98.0	18.0	6.34	17.8	23.6	6.9	1.5	101	1.7
52–48		50 ♂ 65.9	0.45	3.58	0.173	0.069	3.63	1.081	0.173	45.4	92.9	18.2	6.35	19.7	16.1	4.7	1.1	114	1.7
59–154	Very early	22 ♂ 68.2	0.75	6.80	0.235	0.054	2.58	1.059	0.423	39.8	100.0	7.4	0.82†	7.8	23.5	6.9	1.4	106	1.3
60–9	Study done 10 days $\bar{p}$ venesection	60 ♂ 64.1	0.70	5.72	0.271	0.078	2.15	2.634	0.229	68.9	143.7	6.7	3.43	17.5	38.9	11.4	1.6	114	1.7
59–164		55 ♂ 65.7	0.44	2.96	0.194	0.034	3.67	0.978	0.149	56.7	110.8	27.9	3.78	27.3	21.5	6.3	1.2	105	1.7
	Mean	56 63.7	0.58	4.60	0.223	0.056	2.98	1.348	0.225	51.9	109.1	15.6	4.98	18.0	24.7	7.2	1.4	108	1.6
	SD		±0.13							±10.1	±18.3	±7.9		±6.2	±7.6	±2.2	±0.2	±5	±0.5
59–70†		52 ♂ 66.8	0.74	8.84	0.333	0.072	3.44	1.669	0.579	83.5	148.4	10.4		17.4	49.3	14.5	2.6	52	2.1

* See Table I for abbreviations. † Case or value not included in statistics.

daily hemoglobin synthesis, mean erythrocyte life span, and MEEHT. The plasma radioiron curves in these patients may be resolved into three exponential components (Figures 6, 7, 10). Using the slopes and their intercepts, the following average estimates are obtained in patients with endogenous hemochromatosis: 51.9 mg of iron leaves plasma daily $[(\alpha_{12} + \alpha_{15})X_1]$, of which 24.7 mg $(\alpha_{23}X_2)$, or 48 per cent, is used for hemoglobin synthesis, and 18.0 mg per day $(\alpha_{56}X_5)$ goes into stores. The labile storage iron pool is 15.6 mg, approximately twice the plasma iron pool of 7.0 mg. Miscible storage iron in the four patients with advanced hemochromatosis without hemolysis averages 4,980 mg as compared with 820 mg in the young prehemochromatotic patient. Although only 58 per cent of the radioiron is incorporated into circulating erythrocytes, hemoglobin is synthesized at a normal rate of 7.2 g per day or 1.4 g per L per day. Corresponding mean erythrocyte life span is calculated to be 108 days. The estimated MEEHT is 1.6 days.

The compatibility of the predictions of the mathematical models with the experimental results was demonstrated by an analog computer. Intercompartmental rate constants were calculated from observed data using the formulae in Appendix D and inserted into the analog computer in both the "storage exchange" and "normal flow" models. The resultant radioiron curves obtained in plasma, labile erythropoietic, and labile storage compartments in the analog computer reproduce within 5 per cent the observed radioiron curves in plasma, sacral marrow, and liver.

DISCUSSION

Mathematical analysis of the proposed general model (Figure 11a) predicts that tracer radioiron within the plasma iron compartment should decrease with three exponential components when feedback from erythropoietic and storage pools is significant. More than two components are clearly present in the measured plasma radioiron of patients with hemochromatosis in whom significant exchange of iron between plasma and storage compartments is demonstrated by striking hepatic deposition of radioiron (Figures 6, 9, 10). When plasma iron transfer into the storage compartment is negligible, then the general model reduces to the simpler one (Figure 5a) in which exponential decrease of plasma radioiron contains only two components. Measurements of plasma radioiron in normal subjects, in whom hepatic deposition of radioiron is small, demonstrate only two easily distinguishable exponential components, $x_1 = Ae^{-r_1 t} + Ce^{-r_3 t}$ (Figures 1, 2, 5, 14). Patients with complete erythroid aplasia also demonstrate only two components of plasma radioiron; the slope of the second component, however, corresponds in magnitude to r_2, and r_3 is absent (21); plasma radioiron, $x_1 = Ae^{-r_1 t} + Be^{-r_2 t}$ (Figure 15). Using these models, the calculated rates of hemoglobin synthesis, when related to measured total-body hemoglobin, result in estimates of erythrocyte life spans in normal subjects and patients with endogenous hemochromatosis within the previously established normal range of 100 to 130 days (17, 19). There was one exception, a patient (59–70) with endogenous hemochromatosis

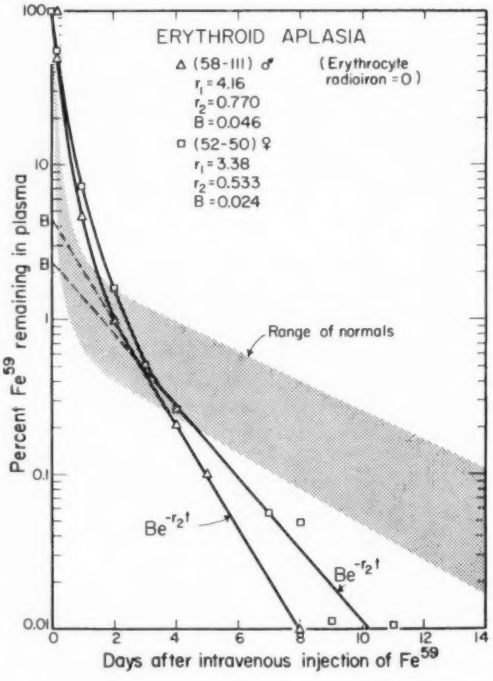

FIG. 15. PER CENT OF RADIOIRON IN PLASMA AFTER INTRAVENOUS INJECTION IN TWO PATIENTS WITH ERYTHROID APLASIA. The initial rapid exponential component is slower than normal while the second exponential storage deposition component is more rapid than the normal erythropoietic fixation component.

whose plasma radioiron remained constant after Day 9 of the study (Figure 12). This corresponds to the "hemolytic" plasma radioiron pattern of Figure 11. The mean erythron life span of this patient with the "hemolytic" pattern, derived from rate of hemoglobin synthesis and measurements of circulating hemoglobin, was reduced to 52 days. All of the other 18 subjects (13 normal, 5 endogenous hemochromatosis) showed continued decrease of plasma radioiron at Day 10 (Figures 2, 7).

Patient 60–9, with endogenous hemochromatosis, was venesected 10 days before the study began. Hemoglobin synthesis in this patient was increased to 11.4 g per day, or 1.6 g per L per day, as compared with 1.3 ± 0.2 g per L per day in normal subjects and 1.4 ± 0.2 g per L per day in the other hemochromatotic patients without hemolysis. Relating this increased rate of hemoglobin synthesis to total hemoglobin (759 g) and its increase during the study (4.8 g per day), a mean erythron life span of 114 days was obtained. This demonstrates increased production of erythrocytes with normal longevity in response to venesection.

Miscible storage iron in the four patients with advanced endogenous hemochromatosis without hemolysis averaged 4,980 mg. Hepatic ferritin, calculated from data obtained in three patients with endogenous hemochromatosis studied by Finch and Finch (23), averaged approximately 2,300 mg. Assuming that the liver contains almost half the total storage iron (30) and that ferritin is distributed in approximate proportion to hemosiderin, the value of 4,980 mg obtained for miscible storage iron is not significantly in excess of estimated total ferritin in these patients. It thus appears that very little of the radioiron deposited in storage is fixed in hemosiderin during the 4 week period following injection of radioiron. Using a hepatic hemosiderin : ferritin ratio of 4.5 derived from the data of Shoden, Gabrio and Finch (35) and Finch and Finch (23) and equating ferritin to miscible storage iron, mean total storage iron of 27,400 mg is calculated for the four patients of this study. Using a hepatic hemosiderin : ferritin ratio of 10.6 derived from the data of Heilmeyer in five patients with hemochromatosis (36), total storage iron of 57,700 mg is calculated for these four patients. Miscible storage iron in the fifth patient, 22 years old and classified as prehemochromatotic, is only 820 mg. Assuming that this corresponds to ferritin, and using a smaller hemosiderin : ferritin ratio of 2.0 (35), calculated storage iron in this patient is only moderately increased to 2,460 mg; using the data of Heilmeyer, 4,670 mg. Miscible storage iron was not calculated in the sixth patient (59–70) with hemolysis, in whom a relatively high equilibrium level of plasma radioiron derived from erythron hemolysis is present (D + E, Figure 12). This is superimposed upon the lower equilibration level of storage pool radioiron with plasma (E, Figure 10) so that storage equilibration could not be measured directly.

The mean erythron life span is calculated as the ratio of total hemoglobin to daily hemoglobin breakdown, assuming that measured erythrocyte kinetics is characteristic of the cells measured throughout their life span. Chromium^{51} and DFP^{32} (P^{32}-diisopropyl fluorophosphate) label the population of circulating erythrocytes. If a population of erythrocytes with skewed distribution of potential life spans is released from the marrow, these cells do not have proportional representation in the circulation. At any given time the circulating blood will contain relatively fewer short-lived erythrons and, conversely, many more long-lived erythrons than does the population originally released from the marrow. If destruction is not entirely random, numerical representation of circulating erythrons becomes weighted in proportion to longevity. On the other hand, the radioiron method presented measures mean erythron life span from the time of fixation of radioiron in maturing erythrons, beginning with the proerythroblast (37, 38). Mean erythron life span measured in this way is comparable with mean survival of the population of circulating erythrons, as determined with the Ashby, DFP^{32} or Cr^{51} techniques, only when erythron destruction is random or occurs with a normal distribution about a definite time interval (21). When defective erythropoiesis results in marrow hemolysis of maturing erythrons or release into the circulation of a skew population, measurement of the circulating erythrons will result in survival times which are considerably longer than the mean erythron life span. A more detailed consideration of these problems together with observations in

patients with hemolytic anemia will be presented elsewhere.

Analysis of earlier ferrokinetic models used for calculating red cell iron turnover and rate of hemoglobin synthesis (1, 2) suggests two reasons for the lack of agreement. The earlier proposal, that rate of iron fixation in erythrocytes is equal to the product of the rate of plasma iron removal and the fraction of radioactive iron found in circulating erythrocytes, implies a linear progression of radioiron from plasma to marrow to red cells without significant feedback. When the return of iron from labile erythropoietic and storage iron pools is neglected, an erroneously large rate of iron fixation in erythrocytes is estimated. In these normal subjects this error averages 52 per cent, while in the patients with endogenous hemochromatosis it averages 22 per cent. Another difficulty arises from equating the maximal fraction of radioiron present in circulating erythrocytes with the maximal fraction of radioiron fixed in maturing erythrons (f). This difference becomes significant in active hemolytic states where maximal *net* incorporation of radioiron into circulating erythrocytes may be as little as 35 per cent (21), without appreciable initial hepatic deposition of radioiron. Under these circumstances, multiplication of plasma iron turnover by maximal *net* fraction of radioiron in circulating erythrocytes results in an erroneously small rate of iron fixation in erythrocytes. If, for example, in a patient with negligible plasma-storage iron exchange and severe hemolytic anemia $(\alpha_{23} + \alpha_{21})/\alpha_{23} = 6/5$ and maximal *net* incorporation of radioiron into circulating erythrocytes is one-third, then iron fixation in erythrons as calculated by multiplying plasma iron turnover by maximal net incorporation of radioiron into circulating erythrocytes would be only 40 per cent ($6/5 \times 1/3 \times 100$ per cent) of the actual iron fixation in erythrons.

In terms of the proposed mathematical models, initial accumulation of radioiron in sacral marrow is largely the result of transfer and equilibration of radioiron between the plasma and labile erythropoietic pools. If this marrow uptake, mirroring disappearance of radioiron from plasma and normally almost complete in 8 hours (Figures 1, 4), represented irreversible intracellular fixation of radioiron, then release of radioiron from marrow and its appearance in circulating erythrocytes would correspond to the division and maturation times of the various stages of erythron development in which iron is fixed. Since total division and maturation time of erythrons is estimated to be 2.5 to 3.0 days (39, 40), marrow release and appearance in circulating erythrocytes of radioiron would be complete in approximately 3 days. The observed time of 7 to 10 days required normally for maximal incorporation of radioiron into circulating erythrocytes (Figure 3) is consequently incompatible with the assumption that initial marrow accumulation of radioiron occurs by irreversible intracellular fixation in marrow erythrons. If, on the other hand, a nonerythropoietic labile pool (also a nonstorage labile pool, as shown by absence of considerable hepatic accumulation of radioiron) is postulated to supply radioiron to marrow via plasma at the appropriate rate, then sacral marrow radioiron accumulation would be much more gradual and reduced than that observed (Figure 4).

The concept of a labile erythropoietic pool is central to the proposed mathematical models. These models were first presented in 1956 (5, 6). At that time no morphologic or biochemical entity corresponding to such a pool had been observed. Bone marrow hemosiderin and ferritin were and still are considered part of the iron storage compartment. Subsequently in 1957, Bessis and Breton-Gorius, using electron microscopy, demonstrated that ferritin may be transferred by micropinocytosis from reticulum cells and extracellular fluid into the cytoplasm of maturing erythrons or, possibly, the reverse sequence occurs (41–43). It is well established that ferritin is a labile form of iron in equilibrium with ionic iron: Fe^{+++} + apoferritin $\rightleftarrows$ ferritin (36, 44–51). Since it is known that plasma iron is bound to transferrin (52, 53), the following over-all equilibrium is present: transferrin − Fe $\rightleftarrows$ transferrin + Fe^{+++} + apoferritin $\rightleftarrows$ ferritin. It seems unlikely, however, that marrow ferritin constitutes the labile erythropoietic pool. Approximately half of the total storage iron is estimated to be present in marrow (30). In normal individuals ferritin and hemosiderin iron are approximately equal; usually slightly more ferritin than hemosiderin is present (23, 35). Marrow ferritin may thus normally represent one-half of total ferritin and one-quarter of total storage iron. Return of ra-

dioiron from miscible storage iron to plasma is quite slow, becoming appreciable only after 14 days (Figure 7). Miscible storage iron is probably composed largely of ferritin, since it corresponds closely in magnitude to estimated total ferritin. Ferritin, then, appears to exchange with plasma relatively slowly, and hence does not exhibit the very high degree of lability of the labile erythropoietic pool. A more probable conjecture is that the labile erythropoietic pool consists of iron reversibly bound to the maturing erythron membrane, thereby being in a position to equilibrate rapidly with plasma, yet being simultaneously available for intracellular fixation in heme synthesis. Sondhaus and Thorell have recently shown by microspectrographic study of individual salamander erythrons that the ratio of nonheme iron (probably Fe^{+++}) to hemoglobin iron exceeded 20 in the youngest cells and was approximately 2 from the time hemoglobin synthesis was one-third complete until maturity (54). Walsh and associates have suggested that the reticulocyte membrane functions as an intermediate link between plasma transport and heme synthesis (55). Jandl, Inman, Simmons and Allen have shown in man that iron bound to transferrin is actively transferred to the reticulocyte membrane, but not to mature erythrocytes (56). After incubation at 37° C for 2 hours of reticulocytes with Fe^{59}-transferrin, approximately half of the reticulocyte Fe^{59} was associated with the reticulocyte membrane as nonheme iron, the other half was present in the cell interior, mostly as heme iron. The uptake of iron by reticulocytes was shown not to be primarily linked to heme synthesis. Pb^{++} (10^{-4} M) blocked iron incorporation into heme without reducing iron transfer, with resultant accumulation of iron on the reticulocyte membranes.

The erythropoietic labile pool, as calculated in this study, is decreased or absent in anemias with erythroid hypoplasia or aplasia (21). This finding would be expected if the erythropoietic labile pool consists of iron bound to membranes of immature erythrons. This hypothesis is also supported by preliminary measurements in our laboratory of canine marrow and human and rabbit reticulocytes (21). In normal dogs, 8 hours after most of the tracer plasma radioiron had accumulated in marrow, approximately half of the marrow radioiron was present as erythron nonheme radioiron. The nonheme radioiron was associated chiefly with the erythron membrane, while most of the heme radioiron was present in the erythron interior. Heme radioiron was determined in human circulating erythrons by measuring the specific activity of doubly crystallized heme. These measurements were made at varying intervals during an iron kinetic study. The percentage of erythron radioiron present in heme was found to be 43 per cent at Day 1, 56 per cent at Day 2 and 98 per cent at Day 14. These values are in close agreement with the independently calculated rate constant for transfer from the labile pool to fixed iron in erythrons, $\alpha_{23} = 0.49$. In an untreated patient with iron deficiency and polycythemia vera this transfer was greatly accelerated, with 78 per cent at 2.7 hours and 94 per cent at 1 day of erythron radioiron present in heme; these values are in agreement with the independently calculated $\alpha_{23} = 17$. Human and rabbit reticulocytes were labeled *in vivo* by transfer of tracer radioiron from plasma transferrin. Incubation of the washed, labeled reticulocytes in plasma for 1 hour resulted in transfer of up to 17 per cent of the reticulocyte radioiron back to plasma (but not to acid citrate dextrose solution), thereby demonstrating the lability of iron in immature erythrons.

The application of this model in normal subjects provides estimates of hemoglobin synthesis which correspond to well accepted values for normal life span of circulating erythrocytes (17–20). Since iron absorption is roughly equivalent to daily iron loss of 1.2 mg per day (57–67), net iron removed from plasma for hemoglobin synthesis, 21.4 mg per day, under steady state conditions [Table I: $\alpha_{12}X_1$ (32.5 mg per day) $- \alpha_{21}X_2$ (11.1 mg per day)] must enter plasma directly or indirectly from destroyed red cells. It follows that—unless the life span of normal *circulating* erythrocytes is significantly shorter than the finite life span well established by previous studies of Ashby, Eadie and Brown, and Pollycove and coworkers (17–20), thereby releasing more than 21 mg iron per day for fixation in maturing erythrons—only insignificant amounts of iron from circulating erythrocytes could bypass plasma (via breakdown of circulating erythrocytes to ferritin within marrow reticulum cells and subsequent

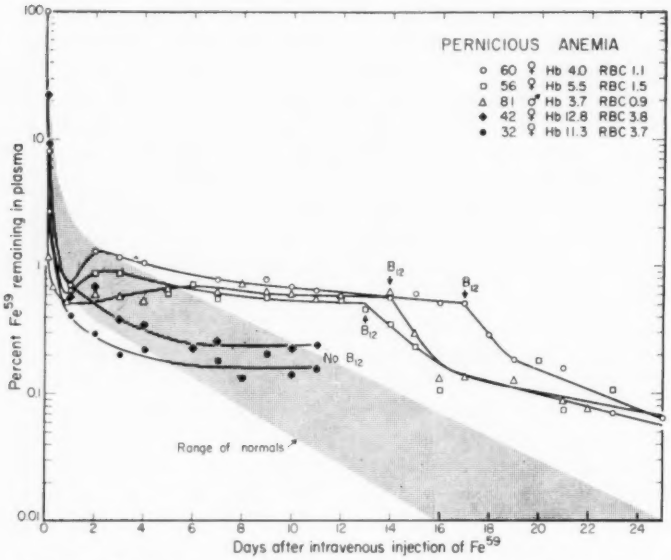

FIG. 16. PER CENT OF RADIOIRON IN PLASMA AFTER INTRAVENOUS INJECTION IN PATIENTS WITH PERNICIOUS ANEMIA. Plasma radioiron of patients with severe anemia in relapse reaches a minimal value within 1 day and subsequently increases. Administration of vitamin B_{12} is followed by rapid and sustained decrease of Fe^{59}. Plasma radioiron of patients with borderline anemia equilibrates at a constant level within 6 days.

direct transfer of this ferritin from cytoplasm of reticulum cells into cytoplasm of immature erythrons) (43). It is conceivable, however, that iron from normal destruction in the marrow of a small fraction of maturing erythrons is complexed to ferritin in marrow reticulum cells, and then reincorporated into other erythrons, *without* entering plasma or labile erythropoietic compartments. This breakdown of hemoglobin during erythron maturation was first suggested by the findings of London, West, Shemin and Rittenberg (68) and Gray, Neuberger and Sneath (69) to explain the early presence in normal subjects of a relatively high specific activity of N^{15}-stercobilin at a time when specific activity of circulating N^{15}-hemin is low; 11 to 20 per cent of total N^{15}-stercobilin being recovered in the initial 8 day period following ingestion of N^{15}-glycine. Other explanations of this finding suggested by these workers are that the stercobilin may be derived from porphyrins not synthesized into hemoglobin or from bile pigment not derived from degradation of a porphyrin ring. When erythron hemolysis occurs in marrow, erythroblastic (or megaloblastic) karyorrhexis and erythrophagocytosis (70–72) and characteristic uniformity of hemosiderin granulation (73) are seen. The erythrophagocytosis results from the death of erythrons. It is doubtful that viable erythrons are phagocytized (71). This occurs in pernicious anemia, erythremic myelosis, refractory anemia with erythroid hyperplasia, and thalassemia major (73, 74). The mean effective erythron hemoglobinization time in these disorders is prolonged markedly (Figure 13), and plasma radioiron stabilizes in 2 days or less at a high equilibrium level (Figures 5b, D; 16); parenteral administration of vitamin B_{12} to patients with pernicious anemia in relapse results in a prompt exponential decrease of plasma radioiron (Figure 16). These observations suggest that there is relatively little shunting of plasma by iron via the pathway: reticulum cell erythrophagocytosis → reticulum cell ferritin → micropinocytosis → erythron ferritin. Two female patients with clinically mild pernicious anemia were studied. The hemoglobin concentrations were 12.8 and 11.3 g per 100 ml; the hematocrits, 39 and 37 per cent, and red blood cells 3.8 and 3.7 million

per mm^3. Plasma radioiron in both cases stabilized at a constant equilibrium level in 6 days (Figure 16), in contrast to the exponential decrease of radioiron for approximately 14 days in all 13 normal subjects (Figures 1, 2, 14). These observations suggest that in normal subjects there is no significant destruction of maturing erythrons with direct reincorporation of all recovered iron, thereby bypassing plasma and labile erythropoietic iron compartments.

There remains the possibility that iron irreversibly fixed in marrow erythrons constitutes the labile erythropoietic pool. If this obtained, then approximately one-third of all iron fixed in erythrons would normally be quickly returned to plasma as a result of destruction of maturing erythrons and only one-quarter of the total number per day ($\alpha_{23} = 0.258$, Table I) would survive to mature and enter the circulation without destruction. This degree of destruction is not compatible with the N^{15}-stercobilin measurements of London (68) and Gray (69) and their colleagues nor with morphologic examinations of normal marrow (70–73). Iron is chelated very tightly to transferrin so that no significant dissociation occurs (52, 53). This precludes the possibility that significant amounts of dissociated Fe^{59} escape through the capillary walls and become bound to extravascular protein. One must consider the possibility that a considerable fraction of Fe^{59}-transferrin transfers into the extravascular fluid and that re-entry of radioiron into plasma occurs from this extravascular pool. Transferrin exchange with extravascular extracellular fluid, however, is relatively slow, the half-time being approximately 30 hours (75, 76). This is quite long compared with 2.5 hours, the longest half-time of initial plasma radioiron decrease observed in patients with hemochromatosis. Less than 10 per cent of the radioiron transfers to extracellular fluid in this patient and approximately only 5 per cent in normal subjects. This is insufficient to explain the prolonged appearance of radioiron in circulating erythrocytes if all erythron radioiron were irreversibly fixed.

Measurements of surface radioactivity in normal subjects show that as radioiron is removed from plasma, hepatic radioactivity usually decreases or, at most, remains constant (Figures 1, 4). Uptake of radioiron in rib marrow and, to a slight extent, vertebral marrow also contributes to the "hepatic" counting rate. This means that, normally, maximal deposition of radioiron in liver is less than radioiron initially present in hepatic plasma. Since liver contains approximately half of the body iron stores (30), and on the assumption that blood comprises approximately 18 per cent of liver (1,500 g) (77, 78), we estimate that maximal iron deposition in stores is less than 10 per cent of total fixation of plasma iron in normal subjects. Measurements of net incorporation of radioiron into erythrocytes are in close agreement with this estimate (Figures 1, 3). The av-

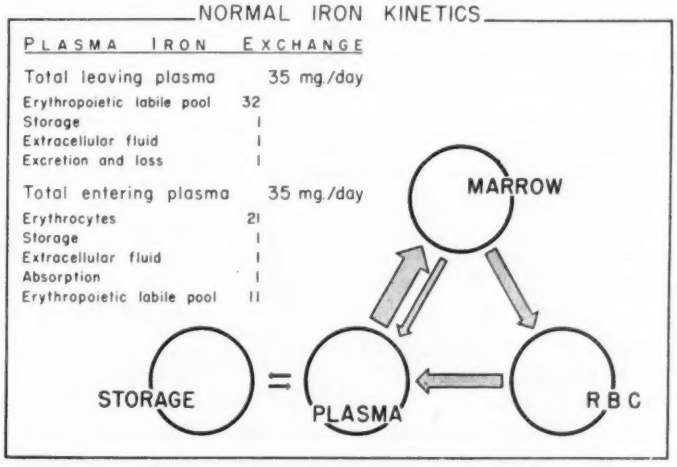

FIG. 17. QUANTITATIVE ASPECTS OF NORMAL IRON KINETICS.

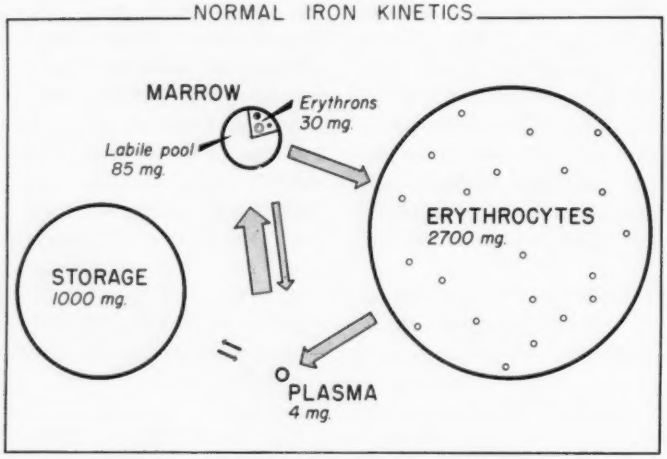

FIG. 18. NORMAL DISTRIBUTION OF IRON IN VARIOUS COMPARTMENTS.

erage daily fixation of iron in maturing erythrons is 21.4 mg per day in normal subjects; storage iron deposition is estimated to be less than one-tenth this amount, or between 0 and 2 mg per day. Relating MEEHT of 1.4 days to daily fixation of iron in maturing erythrons, 21.4 mg per day, results in a total amount of $1.4 \times 21.4 = 30.0$ mg of iron irreversibly fixed in maturing erythrons. From these considerations, the observations and results presented in Tables I and II, and established data concerning body iron stores (47), iron absorption (58–67), iron excretion (57, 60), and plasma globulin exchange with extracellular fluid (75, 76), the main pathways and compartments may be represented as in Figures 17 and 18. Corresponding pathways and compartments in patients with endogenous hemochromatosis without hemolysis are illustrated in Figures 19 and 20.

Mean effective erythron hemoglobinization time is the mean time interval between fixation of radioiron in maturing erythrons and appearance of radioiron in circulating erythrocytes. This is shown in Figures 1, 6, and 13 and calculated in Appendix C. Radioiron enters and is fixed in

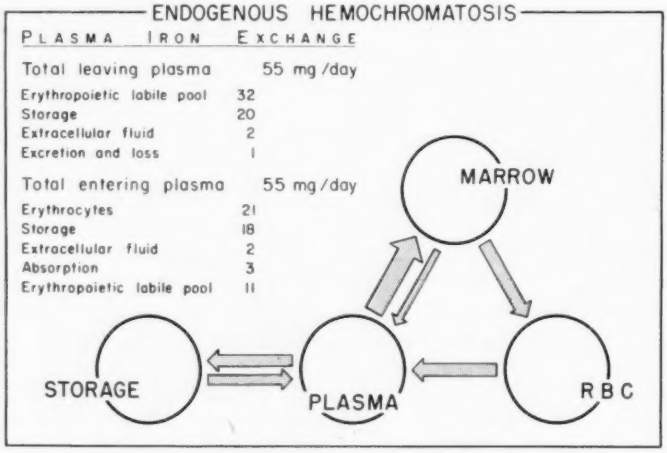

FIG. 19. QUANTITATIVE ASPECTS OF IRON KINETICS IN PATIENTS WITH ENDOGENOUS HEMOCHROMATOSIS.

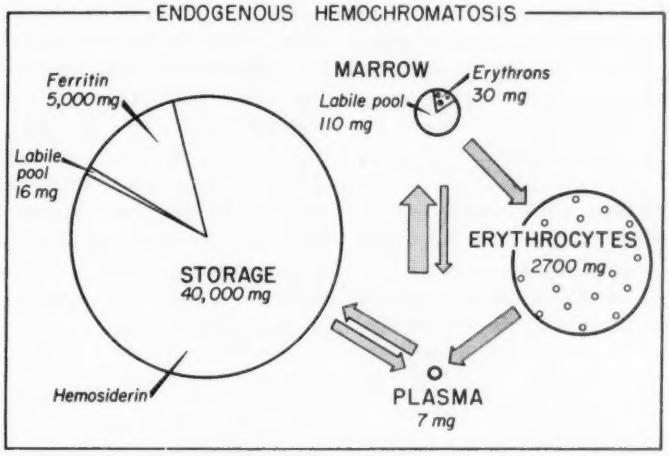

FIG. 20. DISTRIBUTION OF IRON IN VARIOUS COMPARTMENTS IN PATIENTS WITH ENDOGENOUS HEMOCHROMATOSIS.

maturing erythrons at all stages of development. The amount of radioiron per cell is high for young cells and low for older ones (37, 38). However, many more erythrons at the later stages of development are present. Consequently, the mean time intervals from fixation to appearance in circulating erythrocytes (mature erythrons) is probably comparable in magnitude with a mean hemoglobinization time averaged over all stages of development. *Total* division and maturation time for erythrons, 2.5 to 3.0 days, has been estimated from mitotic counts and ratios of cell counts of erythrons of varying maturity (37, 38). Since radioiron enters maturing erythrons at all stages of development, the MEEHT may be expected to be roughly of the order of one-half *total* erythron division and maturation time. MEEHT of 1.4 ± 0.4 days in normal subjects and 1.6 days in patients with endogenous hemochromatosis were calculated. These are approximately one-half the total erythron division and maturation time.

The delay of radioiron incorporation into circulating erythrocytes which is seen in patients with hemochromatosis probably is not the result of hemolysis of maturing erythrons, delayed erythron maturation, or increased intermitotic interval, since MEEHT is normal. The most probable cause of the delay is the increased time required for removal of radioiron from the increased labile iron pool (Figure 13, Appendix C). Conversely, when erythropoiesis is greatly increased, abnormally rapid incorporation of radioiron into circulating erythrocytes probably is not the result of shortened erythron maturation, decreased intermitotic interval, or a decrease in normal destruction of maturing erythrons as suggested by Stohlman (79), since MEEHT is normal (Figure 13, sickle cell anemia) (21). Early delivery into the circulation of a large fraction of reticulocytes and a few nucleated erythrons could conceivably shorten *total* division and maturation time *in the marrow* by as much as a full day. Since radioiron is fixed in maturing erythrons at all stages of development, the *mean* time for radioiron to appear in circulating erythrocytes could be shortened in this way by only one-half or, at the very most, three-quarters of a day. Early release of maturing erythrons cannot be the major factor in reduction of mean time required for appearance of circulating radioiron from 4 days in normal subjects to 2 days in patients with greatly increased erythropoiesis (Figure 13, sickle cell anemia). Rapid incorporation of radioiron into circulating erythrocytes is probably largely the result of rapid fixation of radioiron in maturing erythrons—i.e., α_{23} is increased, in accordance with the proposed mathematical model (Figure 13, sickle cell anemia, Appendix C). When this occurs, and the mean effective erythron hemoglobinization time is normal—i.e., marrow hemolysis is absent—labeling of erythrons may be complete in a fraction of a day, and the time interval between the maximal

net incorporation of radioiron into erythrons and maximal net incorporation into circulating erythrocytes furnishes a good estimate of the time required for the earliest labeled cell, the proerythroblast (37), to divide, mature, and enter the blood. This time interval is approximately 3 days, as shown in Figure 13, sickle cell anemia, and is not appreciably shortened even in the most severe hemolytic anemias (21, 22). These observations are compatible with the concept that compensatory increased erythropoiesis is the result of increased division of erythroid precursors more immature than proerythroblasts, or increased differentiation of multipotential stem cells into erythroid precursors which then proceed to divide and mature in normal fashion, as proposed by Osgood (80), Alpen and Cranmore (81) and Erslev (82).

Markedly increased amounts of N^{15}-stercobilin in the first week after ingestion of N^{15}-glycine (83) and markedly increased marrow erythrophagocytosis (70–72) have been observed in patients with pernicious anemia. These observations indicate that marrow hemolysis of maturing erythrons, not prolonged erythron maturation or increased intermitotic interval, is responsible for the marked increase of mean effective erythron hemoglobinization time (Figure 13). The term, mean *effective* erythron hemoglobinization time, is used because, when hemolysis occurs in marrow, the time interval between initial fixation of some radioiron in maturing erythrons and the appearance of this label in a cell that reaches the circulation may span two or more passages through the marrow maturational pathway (21, 22, 34, 70, 73, 84).

The calculated amounts of miscible storage iron in the patients with endogenous hemochromatosis correspond closely with estimates of total ferritin derived from direct measurements of hepatic ferritin (23, 35). The amount of iron calculated to be present normally in the labile erythropoietic pool (84 mg) lies well within the accepted normal range of 750 to 1,500 mg of total-body nonhemoglobin iron (47). The labile erythropoietic pool was found to be increased to a mean of 117 mg in the 6 patients with endogenous hemochromatosis and reduced markedly in 11 patients with iron-deficiency anemia to a mean of 14 mg (8, 21, 22). In these iron-deficient patients the mean fraction of labile pool turnover fed back to plasma, $\alpha_{21}/(\alpha_{23} + \alpha_{21})$, is 0.066 (8, 21, 22), compared with a normal mean of 0.342. This suggests that the *fraction* of the total amount leaving the labile pool that is not utilized for hemoglobin synthesis is greatly decreased when the pool size is very small. The distribution and movement of iron, as calculated from mathematical application of the proposed model, appears consistent with established data concerning erythrocyte life span, total-body nonhemoglobin iron, erythron division and maturation time, and concepts regarding mass action equilibria.

CONCLUSIONS AND SUMMARY

A mathematical model of iron kinetics is proposed and used in the study of 13 normal subjects and 6 patients with endogenous hemochromatosis. Of central importance to this model is the concept of a labile erythropoietic iron pool interposed between iron bound to transferrin in plasma and iron irreversibly fixed in erythrons for heme synthesis. This model is applied to measurements of radioiron in plasma, circulating red cells, and surface counting rates over liver, spleen, and marrow. Together with measurements of plasma iron concentration and red cell volume, this permits calculation of hemoglobin synthesis, mean erythron life span, mean effective erythron hemoglobinization time, storage iron deposition, and miscible storage iron. Results are presented that are in agreement with established concepts concerning the life span of normal erythrocytes, erythron maturation, and body iron stores. Increased storage iron deposition, miscible storage iron, and plasma iron removal were evident in all hemochromatotic patients. Hemoglobin synthesis and erythron life span were normal in five patients with hemochromatosis; one was found to have compensated hemolysis. Erythron hemolysis, whether in marrow, or subsequent to erythron entry into the circulation, may be detected and quantitated by analysis of plasma radioiron, which, as a result of hemolysis, equilibrates at a constant value in 10 days or less. Evidence is presented suggesting that there is insignificant erythron iron bypass of plasma, either by destruction of maturing erythrons in the marrow or via erythrophagocytosis in marrow of circulating erythrocytes.

APPENDIX A

In this section a mathematical analysis of the kinetics model illustrated in Figure 11a is presented; the slow return to plasma of iron from storage reserve shown in Figure 11b is neglected. The objectives of this analysis are to obtain a qualitative picture of the turnover of iron and to derive equations, based on the turnover of labeled iron in the various compartments, which can be applied to experimental data in order to determine the rate constants describing the ferrokinetic system.

The conditions assumed in this analysis are:

1) The exchange of iron (labeled or unlabeled) between the various compartments obeys first order kinetics. Delay is assumed to be absent, an assumption known to be incorrect for compartment 3, representing the erythropoietic series. If feedback from this compartment or from the mature red cells (compartment 4) does not occur during the course of the experiment, this will not affect predictions for the turnover of labeled iron in the plasma (or other compartments). An empirical method for estimating the delay in the erythropoietic series is presented in Appendix C.

2) Let X_i be the amount of iron (labeled and unlabeled) in compartment i, and x_i the amount of labeled iron in this compartment. Let α_{ij} represent the *rate constant* from compartments i to j. It is assumed the α_{ij}'s have the same value for labeled and unlabeled iron. Further, the α_{ij}'s are assumed to remain constant.

With the above assumptions, the amount of labeled iron leaving compartment i for compartment j per unit time is $\alpha_{ij}x_i$. The equations describing the turnover of radioactive iron in the general model illustrated in Figure 11a follow.

$$\begin{aligned}
dx_1/dt &= -(\alpha_{12}+\alpha_{15})x_1 + \alpha_{21}x_2 + \alpha_{51}x_5\\
dx_2/dt &= \alpha_{12}x_1 - (\alpha_{21}+\alpha_{23})x_2\\
dx_3/dt &= \alpha_{23}x_2 - \alpha_{34}x_3\\
dx_4/dt &= \alpha_{34}x_3\\
dx_5/dt &= \alpha_{15}x_1 - (\alpha_{51}+\alpha_{56})x_5\\
dx_6/dt &= \alpha_{56}x_5
\end{aligned} \qquad [1]$$

The boundary conditions representing injection of labeled iron into the plasma at $t = 0$ are: $x_1(0) = 1$; $x_2(0) = x_3(0) = x_4(0) = x_5(0) = x_6(0) = 0$.

Equations 1 may be solved by using the Laplace transform. With the above boundary conditions, the general solution for compartment 1 is:

$$x_1(t) = Ae^{-r_1t} + Be^{-r_2t} + Ce^{-r_3t}. \qquad [2]$$

For the cases in which significant storage of iron occurs the plasma clearance curve can be resolved into three exponential components in agreement with the above equation. The values of A, B, C, r_1, r_2 and r_3 are described by the following equations.

$$\begin{aligned}
a + b &= A(r_2 + r_3) + B(r_1 + r_3) + C(r_1 + r_2)\\
ab &= Ar_2r_3 + Br_1r_3 + Cr_1r_2\\
c &= r_1 + r_2 + r_3\\
d &= r_1r_2 + r_1r_3 + r_2r_3\\
e &= r_1r_2r_3
\end{aligned} \qquad [3]$$

where

$$\begin{aligned}
a &= \alpha_{51} + \alpha_{56}\\
b &= \alpha_{21} + \alpha_{23}\\
c &= \alpha_{12} + \alpha_{15} + \alpha_{21} + \alpha_{23} + \alpha_{51} + \alpha_{56} = \alpha_{12} + \alpha_{15} + a + b\\
d &= \alpha_{12}(\alpha_{23} + a) + \alpha_{15}(\alpha_{56} + b) + ab\\
e &= \alpha_{12}\alpha_{23}a + \alpha_{12}\alpha_{56}b.
\end{aligned} \qquad [4]$$

The values a, b, c, d, and e can be determined from the experimental plasma iron curve, using Equations 3. These values can then be introduced into Equation 4 to solve for the α's. However, there are six unknowns and only five equations. An additional equation dependent upon the α's describes the fraction f of labeled iron that appears in red cells at the end of the initial phase of incorporation—i.e., at approximately 2 weeks, when red cell incorporation of radioiron from storage reserve is still negligible.

$$f = \frac{(\alpha_{23}/b)\alpha_{12}}{(\alpha_{23}/b)\alpha_{12} + (\alpha_{56}/a)\alpha_{15}}. \qquad [5]$$

Solution of Equations 4 and 5 yields values for the different α's. The method of data reduction is described in Appendix D.

The model for normal iron kinetics illustrated in Figure 5 is a special case of this more general model. The amount of iron going to stores is very small. In this case $\alpha_{15} \simeq 0$. The predicted plasma iron curve reduces to a sum of two exponentials.

$$x_1(t) = Ae^{-r_1t} + Ce^{-r_3t}. \qquad [6]$$

The values of A, C, r_1 and r_3 are related to the rate constants by the following equations.

$$\begin{aligned}
Ar_3 + Cr_1 &= \alpha_{21} + \alpha_{23}\\
r_1 + r_3 &= \alpha_{12} + \alpha_{21} + \alpha_{23}\\
r_1r_3 &= \alpha_{12}\alpha_{23}.
\end{aligned} \qquad [7]$$

The method of solution of Equations 7 to determine values for the rate constants is described in Appendix D.

APPENDIX B

Calculation of hemoglobin synthesis, iron deposition and mean erythron life span

1. Hemoglobin synthesis. Iron irreversibly fixed each day in maturing erythrons for hemoglobin synthesis is equal to the product of the amount of iron in the labile erythropoietic pool (X_2) and the *fraction* of this pool irreversibly fixed each day in maturing erythrons (α_{23}). Fe for Hb synthesis (RBC Fe turnover) = $\alpha_{23}X_2$ mg/day.

$X_2 = \alpha_{12}X_1/b$ [since $\alpha_{12}X_1 = (\alpha_{23} + \alpha_{21})X_2$; and $b = \alpha_{23} + \alpha_{21}$]

X_1 = amount of iron in plasma

= [plasma Fe conc. (μg/ml) × plasma vol (ml)/ 1,000 (μg/mg)] mg.

Assuming negligible nonhemoglobin iron in mature erythrocytes and since 1 g hemoglobin contains 3.4 mg iron,

daily hemoglobin synthesis = $(\alpha_{23}X_2/3.4)$ g.

2. Iron deposition. Iron deposition in relatively fixed stores is equal to the product of the amount of iron in the labile storage pool (X_5) and the *fraction* of this pool deposited each day in relatively fixed storage form (α_{56}).

Iron deposition $= \alpha_{56}X_5$ mg/day.

$$X_5 = \alpha_{15}X_1/a \quad [\text{since } \alpha_{15}X_1 = (\alpha_{56} + \alpha_{51})X_5; \text{ and } \quad a = \alpha_{56} + \alpha_{51}].$$

3. Miscible storage iron. The constant fraction of radioiron in plasma gradually reached after 2 weeks, E, may be viewed as the result of mixing a fraction of radioiron, $1 - f$, initially in plasma iron, X_1, with a miscible storage pool of iron, X_{ms}.

$$1 - f = \frac{E}{X_1}(X_1 + X_{ms})$$

$$X_{ms} = \frac{(1 - f)X_1}{E} - X_1 = \frac{(1 - f - E)X_1}{E} \text{ mg}$$

$$\simeq \frac{(1 - f)X_1}{E} \text{ mg} \quad (\text{since } 1 - f \gg E).$$

4. Mean erythron life span. Mean erythron life span = [total hemoglobin (g)/daily hemoglobin loss (g/day)] days:

a) When total hemoglobin remains constant—i.e., red cell volume remains constant during the period of study—daily Hb loss = daily Hb synthesis;

b) When total hemoglobin is decreasing—i.e., initial red cell volume is greater than final red cell volume—daily Hb loss = daily Hb synthesis + daily total Hb decrement (ΔHb_d);

c) When total hemoglobin is increasing—i.e., initial red cell volume is less than final red cell volume—daily Hb loss = daily Hb synthesis − daily total Hb increment (ΔHb_i). So that for all cases (a, b, c):

Mean erythron life span

$$= \frac{\text{total hemoglobin (g)}}{\text{daily hemoglobin synthesis} + Hb_d \text{ (g/day)} - Hb_i} \text{ day}$$

where

$$Hb_d = \frac{(RCV_0 - RCV_f)MCHC/100}{t} \text{ g/day}$$

$$Hb_i = \frac{(RCV_f - RCV_0)MCHC/100}{t} \text{ g/day}$$

RCV_0 = initial red cell volume (ml); RCV_f = final red cell volume (ml); MCHC = mean corpuscular hemoglobin concentration (per cent); t = days between initial and final red cell volume measurements.

APPENDIX C

Calculation of mean effective erythron hemoglobinization time

The time interval between incorporation of iron into marrow erythrons and its appearance in circulating erythrocytes is designated as the mean effective erythron hemoglobinization time (MEEHT). Cumulative incorporation of radioiron into maturing erythrons is calculated from the intercompartmental rate constant α_{23} and the maximal net incorporation of radioiron into erythrocytes, N_{max}. N_{max}, the *net* fraction of radioiron involved in erythropoiesis is equal to the total fraction involved in erythropoiesis, f, only when hemolysis is absent. N_{max} is partitioned between a) the combined plasma and labile erythropoietic compartments, and b) the maturing erythron compartment. Since the plasma compartment is usually very small (3 to 5 per cent) relative to the labile erythropoietic compartment, the fraction of the labile erythropoietic compartment fixed each day in maturing erythrons is approximately the same as the fraction of the *combined* plasma and labile erythropoietic compartments fixed each day in maturing erythrons; i.e.,

$$\alpha_{23} \simeq \frac{X_2}{X_1 + X_2}\alpha_{23}.$$

Whenever X_2 is not much greater than X_1, then the term $X_2/(X_1 + X_2)\alpha_{23}$ should be substituted for α_{23} in the following equations. If labile radioiron for erythropoiesis (in plasma and labile erythropoietic pools) is designated as L, and radioiron irreversibly fixed in maturing erythrons is designated as F and net incorporation of radioiron into circulating erythrocytes is designated as N, then,

$$dL/dt = -\alpha_{23}L$$

$$dL/L = -\alpha_{23}\,dt$$

$$\log L = -\alpha_{23}t + C$$

$$L = C'e^{-\alpha_{23}t}, \text{ since } L = N_{max} \text{ at } t = 0,$$

$$L = N_{max}e^{-\alpha_{23}t}, \text{ since } F + L = N_{max}, \text{ then}$$

$$F = N_{max} - N_{max}e^{-\alpha_{23}t}$$

$$F = N_{max}(1 - e^{-\alpha_{23}t}).$$

Using this relationship F can be determined for all days of the study.

MEEHT $= t(N) - t(F)$ days.

Usually a nearly constant maximal time interval has been shown to exist between radioiron net incorporation into erythrons (F) and net incorporation into erythrocytes (N) only in the region of 10 to 30 per cent N_{max}. As incorporation continues, the time interval narrows progressively (Figures 1, 6). This is to be expected, since radioiron is fixed at all stages of erythron maturation and the half-time for transfer of iron from the erythropoietic labile pool to maturing erythrons is of the same order of magnitude as erythron maturation time: approximately 3 days from early proerythroblast (early rubroblast) to circulating reticulocyte (34). Under these circumstances, the best indication of MEEHT is to observe the time required for a small amount of radioiron fixed in erythrons in a relatively short time to emerge into the circulation. In normal individuals and in many pathologic conditions this time interval is maximal and nearly constant between the levels of 10 to 30 per cent N_{max}. For this reason, 20 per cent N_{max} is chosen as a convenient level for measuring MEEHT.

Since

$$F = N_{max}(1 - e^{-\alpha_{23}t})$$
$$N_{max}/X = N_{max}(1 - e^{-\alpha_{23}t_{1/X}(F)})$$
$$e^{-\alpha_{23}t_{1/X}(F)} = 1 - (1/X)$$
$$t_{1/X}(F) = \frac{\log_e [X/(X-1)]}{\alpha_{23}}$$
$$t_{1/5}(F) = \frac{0.223}{\alpha_{23}} \quad t_{1/2}(F) = \frac{0.693}{\alpha_{23}}$$

In some cases, however, when the erythropoietic labile pool is very small, as in iron deficiency, or when erythron fixation of iron is greatly increased, the intercompartmental rate constant α_{23} is greatly increased and most of the radioiron is transferred from the labile pool to maturing erythrons in a time which is very short compared with erythron maturation (Figure 13, sickle cell anemia, pernicious anemia) (22). Under these circumstances the time interval between N and F progressively increases as incorporation continues and the 50 per cent N_{max} level is chosen to measure MEEHT. Usually the time interval at $t_{1/5}(F)$ is used, but for values of $\alpha_{23} > 0.4$, the time interval at $t_{1/2}(F)$ may be greater than at $t_{1/5}(F)$. In borderline cases MEEHT is calculated at both 20 and 50 per cent levels, the larger value being used:

$$\text{MEEHT} = t_{1/5}(N) - t_{1/5}(F)$$
$$\text{or} \quad \text{whichever is larger}$$
$$t_{1/2}(N) - t_{1/2}(F)$$

where $t_{1/X}(N)$ and $t_{1/X}(F)$ are the times for incorporation of N_{max}/X radioiron into erythrocytes and erythrons, respectively; and N_{max} = maximal net incorporation of radioiron into circulating erythrocytes; $t_{1/5}(N)$ and $t_{1/2}(N)$ are obtained directly from the erythrocyte radioiron net incorporation curve.

$$t_{1/5}(F) = 0.223/\alpha_{23}$$
$$t_{1/2}(F) = 0.693/\alpha_{23}.$$

When X_2 is not $\gg X_1$, $X_2/(X_1 + X_2)\alpha_{23}$ is used in the above equation in place of α_{23}.

Examples

a) Endogenous hemochromatosis (Figure 13) (52–48).

$$\alpha_{23} = 0.173$$

Since α_{23} is considerably less than 0.4

$$\text{MEEHT} = t_{1/5}(N) - t_{1/5}(F) \text{ days}$$
$$t_{1/5}(F) = 0.223/\alpha_{23} = 1.3 \text{ days}$$

The net incorporation of radioiron into erythrocytes, Figure 13 (hemochromatosis), $N_{max} = 0.45$ and $N_{max}/5 = 0.09$ with corresponding $t_{1/5}(N) = 3.0$ days.

$$\text{MEEHT} = 3.0 - 1.3 = 1.7 \text{ days}$$

To visualize the relationship of this time interval to the F and N curves, the F curve may be quickly plotted using $t_{1/5}(F)$ and $t_{1/2}(F) = 0.693/0.173 = 4.0$ days and calculating $t_{3/4}(F)$ and $t_{9/10}(F)$ as follows:

$$t_{3/4}(F) = \frac{\log_e X/(X-1)}{\alpha_{23}} = \frac{\log_e 1.33/0.33}{\alpha_{23}}$$
$$= \frac{\log_e 4}{\alpha_{23}} = \frac{1.39}{0.173} = 8.0 \text{ days}$$

$$t_{9/10}(F) = \frac{\log_e 1.11/0.111}{\alpha_{23}} = \frac{\log_e 10.0}{\alpha_{23}}$$
$$= \frac{2.30}{0.173} = 13.3 \text{ days.}$$

b) Sickle cell anemia (Figure 13).

$$\alpha_{23} = 4.74$$

Since α_{23} is considerably greater than 0.4

$$\text{MEEHT} = t_{1/2}(N) - t_{1/2}(F)$$
$$t_{1/2}(F) = 0.693/4.74 = 0.15 \text{ days.}$$

The net incorporation of radioiron into erythrocytes, Figure 13 (sickle cell anemia), $N_{max} = 0.48$ and $N_{max}/2 = 0.24$ with corresponding $t_{1/2}(N) = 1.45$ days.

$$\text{MEEHT} = 1.45 - 0.15 = 1.3 \text{ days.}$$

To plot F,

$$t_{1/5}(F) = 0.223/4.74 = 0.05 \text{ days}$$
$$t_{1/2}(F) = 0.693/4.74 = 0.15 \text{ days}$$
$$t_{3/4}(F) = 1.39/4.74 = 0.29 \text{ days}$$
$$t_{9/10}(F) = 2.30/4.74 = 0.49 \text{ days.}$$

c) Pernicious anemia (Figure 13).

$$\alpha_{23} = 3.57$$

Since α_{23} is considerably greater than 0.4

$$\text{MEEHT} = t_{1/2}(N) - t_{1/2}(F)$$
$$t_{1/2}(F) = 0.693/3.57 = 0.19 \text{ days.}$$

The net incorporation of radioiron into erythrocytes, Figure 13 (pernicious anemia), $N_{max} = 0.14$; $N_{max}/2 = 0.07$ with corresponding $t_{1/2}(N) = 5.2$ days.

$$\text{MEEHT} = 5.2 - 0.2 = 5.0 \text{ days.}$$

To plot F,

$$t_{1/5}(F) = 0.223/3.57 = 0.06$$
$$t_{1/2}(F) = 0.693/3.57 = 0.19$$
$$t_{3/4}(F) = 1.39/3.57 = 0.39$$
$$t_{9/10}(F) = 2.30/3.57 = 0.65.$$

APPENDIX D

*Calculations using iron kinetics models**

Storage exchange (Figure 11)

(Appendix A)

$r_1 = 0.693/t_{1/2}$ of first slope (days) or, more conveniently

$= 16.67/t_{1/2}$ of first slope (hrs)

$r_2 = 0.693/t_{1/2}$ of second slope (days)

$r_3 = 0.693/t_{1/2}$ of third slope (days)

$A = 1 - (B + C)$

B = intercept of second slope

C = intercept of third slope

$c = r_1 + r_2 + r_3$

$d = r_1r_2 + r_1r_3 + r_2r_3$

$e = r_1r_2r_3$

f = max. fraction of injected Fe^{59} incorporated into erythrons

When hemolysis is absent,

f = max. fraction of injected Fe^{59} present in circulating erythrocytes

$a + b = A(r_2 + r_3) + B(r_1 + r_3) + C(r_1 + r_2)$

$ab = A(r_2r_3) + B(r_1r_3) + C(r_1r_2)$

(The latter two simultaneous equations may be solved using the binomial theorem:

$$x = \frac{-L \pm \sqrt{L^2 - 4KM}}{2K}$$

where $Kx^2 + Lx + M = 0$).

Choose roots so that $a > b$. Then,

$$\alpha_{12} = \frac{d - ef/a - e(1 - f)/b - b(c - b)}{a - b}$$

$\alpha_{13} = c - a - b - \alpha_{12}$

$\alpha_{23} = ef/a\alpha_{12}$

$\alpha_{21} = b - \alpha_{23}$

$\alpha_{56} = e(1 - f)/b\alpha_{13}$

$\alpha_{51} = a - \alpha_{56}$

"Normal" flow (Figure 5)

(Appendix A)

Where no appreciable storage deposition (best checked by *in vivo* counting over liver) of Fe^{59} occurs, apply the normal model as follows:

$r_1 = 0.693/t_{1/2}$ of first slope (days)

or

$= 16.67/t_{1/2}$ of first slope (hrs)

r_2 = (omitted in this case)

$r_3 = 0.693/t_{1/2}$ of third slope (days)

A = (not needed in this case)

B = (neglected in this case)

C = intercept of third slope

$b = r_2 + C(r_1 - r_3)$

$c = r_1 + r_3$

$e = r_1r_3$

Then,

$\alpha_{12} = c - b$

$\alpha_{23} = e/\alpha_{12}$

$\alpha_{21} = b - \alpha_{23}$

* Notation refers to kinetics models shown in Figures 5 and 11.

APPENDIX D—*Continued*

Calculation of hemoglobin synthesis

(Appendix B)

Using α's calculated from applicable model,

X_1 = amount of Fe in plasma compartment (mg)

$$= \frac{\text{plasma Fe } (\mu g/ml) \times \text{plasma vol (ml)}}{1{,}000\ (\mu g/mg)}$$

X_2 = amount of Fe in labile erythropoietic pool (mg)

$= \alpha_{12} X_1/b$

Fe to hemoglobin

or (mg/day) $= \alpha_{23} X_2$

RBC Fe turnover

Daily hemoglobin synthesis (g) $= \alpha_{23} X_2/3.4$

Calculation of storage iron deposition

(Appendix B)

X_5 = amount of Fe in labile storage (mg)

$= \alpha_{15} X_1/a$

Fe to storage deposition (mg/day) $= \alpha_{56} X_5$

Calculation of miscible storage iron

(Appendix B)

$X_{ms} = [(1 - f)X_1/E]$ mg

Calculation of mean erythron life span

(Appendix B)

$$\text{Mean erythron life span (steady state condition)} = \frac{\text{total-body hemoglobin}}{\text{daily hemoglobin synthesis}}$$

Mean erythron life span (nonsteady state condition)

$$= \frac{\text{total-body hemoglobin}}{\text{daily Hb synthesis} + \text{daily total Hb decrement}}$$

or

$$- \text{daily total Hb increment}$$

Calculation of mean effective erythron hemoglobinization time

(Appendix C)

MEEHT $= t_{1/5}(N) - t_{1/5}(F)$

or whichever is larger

$t_{1/2}(N) - t_{1/2}(F)$

$t_{1/5}(N)$ and $t_{1/2}(N)$ are obtained from net incorporation of radioiron into circulating erythrocytes curve

$t_{1/5}(F) = 0.223/\alpha_{23}$ $t_{1/2}(F) = 0.693/\alpha_{23}$

When X_2 is not $\gg X_1$, $X_2/(X_1 + X_2)\alpha_{23}$ is used in place of α_{23}

APPENDIX E

Estimation of "f" when both significant hemolysis and plasma-storage iron exchange are present.

Hepatic radioiron counting rates are used as a rough index of storage iron deposition. For patients with hemochromatosis *without* hemolysis, f equals maximal net incorporation of radioiron into erythrocytes. Since hepatic radioactivity is expressed as cpm/cpm$_0$ (hepatic ratio), the fraction of radioiron deposited in storage (chiefly in liver *and* bone marrow), $1 - f$, results in an *increase* in hepatic ratio approximating $(\text{cpm}_{max}/\text{cpm}_0) - 1$. Maximally, only a fraction (f) of all radioiron is in circulating blood, so that a small error is involved in subtracting 1 instead of an amount slightly <1. If all radioiron were deposited in storage, then the calculated *increase* in hepatic ratio would be approximately $\frac{(\text{cpm}_{max}/\text{cpm}_0) - 1}{1 - f}$. Results obtained in the five patients without hemolysis are shown

TABLE III

Calculation of hepatic ratio increment for 100 per cent storage of radioiron in five patients with hemochromatosis and normal erythrocyte life span *

Patient	Maximum hepatic (cpm/cpm$_0$) -1	$1 - f$	Hepatic ratio increment for 100% storage of radioiron $[(\text{max cpm/cpm}_0) - 1]/(1 - f)$
53–57	0.94	0.43	2.2
52–48	1.80	0.55	3.3
59–154	0.75	0.25	3.0
60–9	0.90	0.30	3.0
59–164	0.96	0.56	1.7
Mean of five patients			2.6
Mean of 14 patients with severe erythroid hypoplasia or aplasia (21)			2.6 (range 1.7–4.4)

* Calculation and application are discussed in Appendix E. For comparison, results in other patients without significant hemolysis are also shown.

in Table III. These measurements were made with a 1.75-inch diameter scintillation crystal recessed 1.4 inches in lead shielding, 2.5 inches thick, placed as shown in Appendix F. Mean hepatic ratio increase for 100 per cent storage of radioiron is 2.6 as compared with 2.6 in 14 other patients with severe erythroid hypoplasia or aplasia in which hemolysis was slight or absent. Marked hepatomegaly and obesity result in lower values; lean individuals without hepatomegaly have high values. A hepatic ratio increase for 100 per cent storage of radioiron of 3.0 was estimated for Patient 59–70, since he is lean, with only slight hepatomegaly. His measured maximal hepatic ratio is 1.78 (Figure 12). Since 100 per cent Δ hepatic ratio

$$= \frac{(\text{cpm}_{max}/\text{cpm}_{o}) - 1}{1 - f} = 3.0$$

$$1 - f = \frac{1.78 - 1}{3.0} = 0.26$$

$$f = 0.74.$$

Measured net incorporation of radioiron into circulating erythrocytes at 10 days is 0.63. The relatively moderate decrease of net incorporation of radioiron into erythrocytes, from the calculated value of f of 0.74 to the observed net incorporation of 0.63, is consistent with the moderately shortened calculated mean erythron life span of 52 days as judged by 1) theoretical estimation that a 10 per cent decrease of maximal net erythrocyte incorporation corresponds to daily breakdown of 1.5 to 3.5 per cent of newly formed erythrocytes, and 2) observations in many patients with varying degrees of hemolysis and negligible storage iron deposition (21).

Similar analyses, using splenic ratios derived in patients with erythropoiesis occurring chiefly in spleen or in patients with intense splenic sequestration of erythrocytes, are helpful as a check for estimating f in cases involving both considerable plasma-storage iron exchange and splenic sequestration of erythrocytes. In comparing hepatic and splenic counting rates with sacral counting rates obtained with broad collimation of a 1.75- to 2-inch crystal, it must be kept in mind that the sacral area and adjoining ileum are estimated to represent roughly one-eighth of active marrow, while liver represents roughly one-half of iron storage (35) and is at an average greater distance from the crystal. A factor of approximately one-half should be used for multiplication of hepatic ratio *increments* as an index of storage for comparison with sacral ratio *increments* (when red marrow volume is not significantly increased) as an index of erythropoiesis. Similar considerations for spleen lead to the use of factors ranging from one-half to one-quarter for comparison of splenic erythropoiesis or splenic sequestration with marrow erythropoiesis (21); enlargement of spleen results in decreased splenic counting rate splenic radioiron remaining constant.

APPENDIX F

Placement of scintillation counters for surface measurement[1]

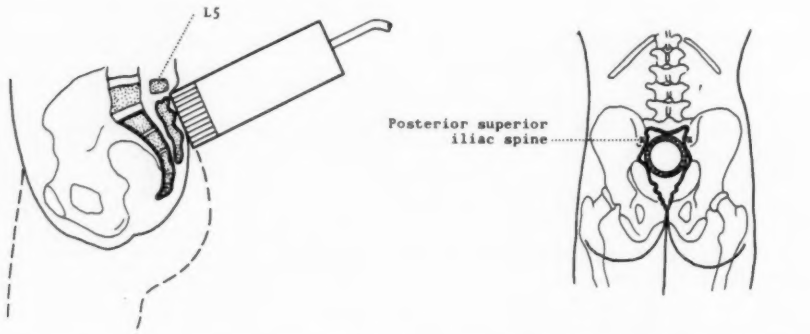
L5
Posterior superior iliac spine
Sagittal section through pelvis showing sacrum and position of scintillation detector.
Posterior surface projection of sacrum showing position of scintillation detector.

FIG. 21. EXTERNAL COUNTING OF SACRUM.

[1] For positions of detector head for external counting of spleen, liver, and sacrum, see Figures 21 and 22.

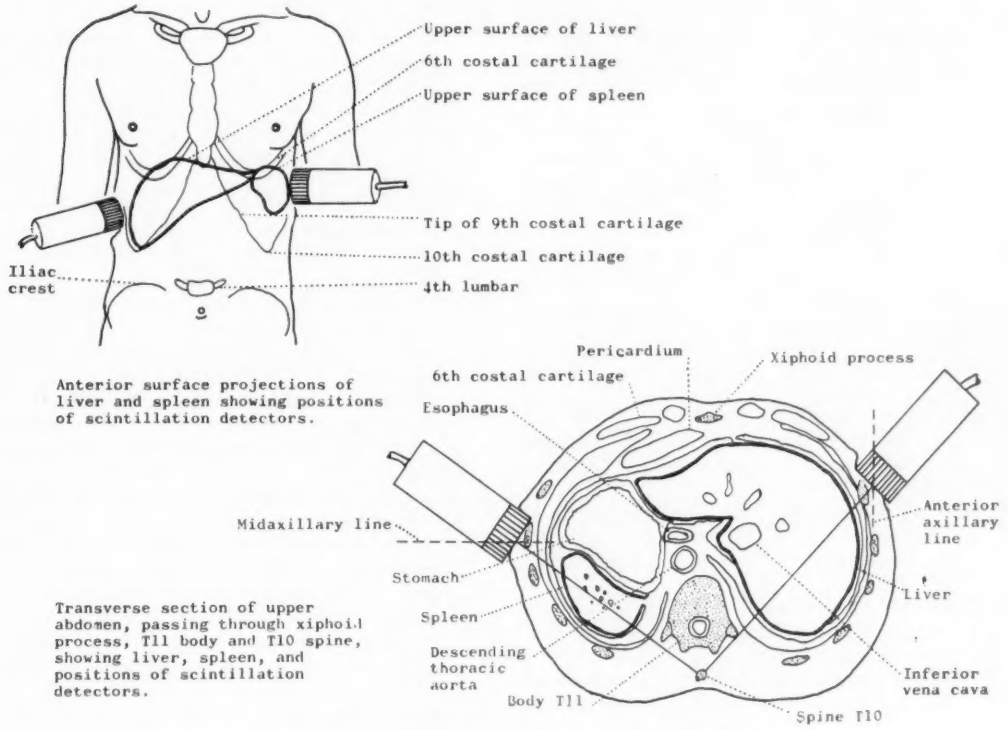

FIG. 22. EXTERNAL COUNTING OF LIVER AND SPLEEN.

Surface landmarks: Usual vertebral levels in quiet respiration

	Vertebrae	
	Spines	Bodies
Xiphisternal joint	T10	T11
Diaphragm—Right dome	T9–10	T10–11
Left dome	T10–11	T11–12

	Spines or lower borders of bodies
Transpyloric plane	L1
Tips of ninth costal cartilages	
Subcostal plane	L3
Supracristal plane	L4
Umbilicus	L3–5
Intertubercular plane	L5
Interspinous plane	S2
Posterior superior spines of ilia	S2
Sacroiliac joint	S1, 2 and 3
Spine of ischium	
Center of acetabulum	Tip of coccyx
Upper margin of symphysis pubis	

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CORRECTION

On page 27 of the paper entitled "Quantitative studies of nasal carriers of staphylococci among hospitalized patients" by Arthur White (J. clin. Invest. 1961, **40**, 23), the parenthetical expression in the footnote to Table VI should read (χ^2:$p < 0.01$). The p value was given incorrectly as < 0.1.

On page 491 of the article entitled "Effects of infusions of serum albumin on serum lipids and lipoproteins in nephrosis" by James H. Baxter, Howard C. Goodman and James C. Allen (J. clin. Invest. 1961, **40**, 490), the subheading under "Plasma volume" in Table I should be "I/II" instead of "II/I."

SIDE CHAIN STEREOISOMERISM AND ANTISTAPHYLOCOCCAL POTENCY OF PENICILLINS *

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Penicillins may be regarded as acylation products of 6-aminopenicillanic acid (Figure 1). This bicyclic dipeptide results from cyclization of L-cysteinyl-L-valine (1) and is loosed into the culture medium in good yield when a strain of *Penicillium chrysogenum* (W.51.20) is cultured in the absence of side chain precursors suitable for acylation at the 6-amino grouping (2). With the attainment of commercially feasible fermentive production of 6-aminopenicillanic acid, the organic chemist can now carry out industrially practical terminal synthesis of known or new penicillins.

Alpha-phenoxyethylpenicillin [penicillin B, 6-(α-phenoxypropioamido)-penicillanate, or phenethicillin—see Figure 1], the first marketed product of such industrial terminal penicillin synthesis, has not been identified as a naturally occurring penicillin. Special biological properties have been ascribed to this compound which also distinguish it from other penicillins (3–5). These include: *1*) resistance to acid (gastric) degradation; *2*) excellent absorption from the gastrointestinal tract; *3*) greater antibacterial potency. Slow penicillinase inactivation quite reasonably would be reflected in apparent *in vitro* superiority in antibacterial effectiveness against penicillinase-producing bacteria. However, enhanced antibacterial effectiveness against nonpenicillinase-elaborating bacteria would require other explanation.

In part, the augmented antibacterial potency claimed for α-phenoxyethylpenicillin has been ascribed to the stereoisomerism inherent in the α-phenoxyethyl side chain characteristic of this penicillin. The L-isomer was reported to be generally more active than the D-isomer, and the racemate [1] more effective than either enantimorph (3). In addition, both L- and DL- forms were alleged to be superior to phenoxymethylpenicillin (V) in antistaphylococcal effectiveness (3).

Aminocarboxybutylpenicillin (penicillin N, synnematin B, cephalosporin N), a fermentation product of certain *Cephalosporium* species, is a penicillin of natural occurrence (7). In the side chain characteristic of this compound there is, also, an asymmetric carbon atom (see Figure 1); the structural configuration of this side chain α-aminoadipic acid moiety is D- (8).

The influence of side chain optical activity upon the antibacterial potency of penicillins can be assessed thoroughly with α-phenoxyethylpenicillin, since L-, D- and DL- mixtures are all available. Useful comparison of potency data regarding α-phenoxyethylpenicillins with other penicillins requires simultaneous assay of the antibacterial potencies of all of the penicillins under consideration, using identical test conditions. Accordingly, the antistaphylococcal effectiveness of L-, D- and racemic α-phenoxyethylpenicillin was tested at the same time as benzylpenicillin (penicillin G), phenoxymethylpenicillin and aminocarboxybutylpenicillin, using a broth tube-dilution method.

Tellurite-positive (9) staphylococci, isolated from clinical specimens, were used for testing. Staphylococci are notorious for many reasons and were chosen for evaluation of the potency of penicillins not only because of the clinical challenge presented by staphylococcal infections, but also because a spectrum of intensity of exposure to penicillinase would be assured. Any large group of staphylococci of recent clinical isolation is cer-

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[1] A product of an organic chemical synthesis, α-phenoxyethylpenicillin should be 50 per cent levo- and 50 per cent dextrorotary, with regard to side chain optical activity. However, federal law requires commercial α-phenoxyethylpenicillin to have at least 55 per cent L-isomer to a maximum of 75 per cent L-isomer (6). *Racemate, raceme,* and *racemic,* when applied to α-phenoxyethylpenicillin in this article, will refer to DL-α-phenoxyethylpenicillin of legal constitution.

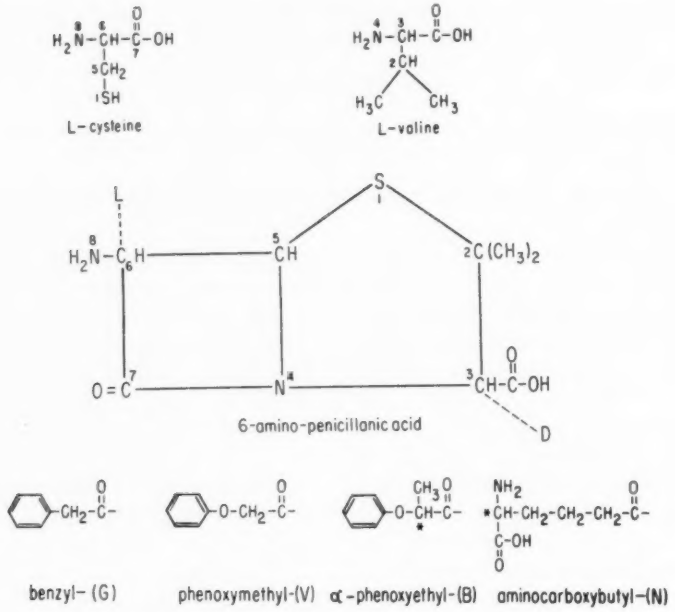

FIG. 1. 6-AMINOPENICILLANIC ACID (6-APA), AVAILABLE VIA BIOSYNTHESIS, RESULTS FROM CYCLIZATION OF THE DIPEPTIDE, L-CYSTEINYL-L-VALINE (1). 6-APA is an optically active compound, with configuration at carbon atoms 3 and 6 as indicated, which has but a low order of antibacterial potency. Both structural and optical configuration must remain intact for any derived penicillin to have a high order of antibacterial activity. Penicillins can be derived from 6-aminopenicillanic acid by acylation of the L-cysteine moiety ($^{5}_{N}$). The acyl radicals indicated are precursor to the side chains which characterize the penicillins studied. Both aminocarboxybutyl- and α-phenoxyethyl- have optically active carbon atoms, indicated by asterisks. The former, in fermentation-produced penicillin N, is levorotary; the latter, in penicillin B, a product of terminal organic synthesis, is available as a quasi-racemate.[1]

tain to include strains capable of penicillinase elaboration—these are the penicillin-resistant staphylococci of clinical significance (10). Penicillinase production appears to vary quantitatively among staphylococcal strains which are competent in this regard. There are penicillin-resistant staphylococci which have not been shown to elaborate penicillinase and, of course, those staphylococci which are markedly susceptible to penicillins do not make penicillinase. Techniques for detection of penicillinase activity are simple and sensitive; assay for this means of penicillin resistance was carried out with all of the staphylococci under study, using benzylpenicillin, phenoxymethylpenicillin and racemic α-phenoxyethylpenicillin.

MATERIALS AND METHODS

Staphylococci. Human associated staphylococci of community-wide origin (Salt Lake City) were used in testing. The total of 200 tellurite-positive (9) isolates were obtained by culture of: *1*) lesions acquired by patients in the course of hospitalization, 50 isolates; *2*) anterior nares of normal hospital personnel, 50 isolates; *3*) lesions acquired outside the hospital, cultures taken prior to any antibacterial therapy, 50 isolates; *4*) anterior nares of normal high school seniors, 50 isolates. Details regarding collection and storage of these isolates are the same as those given in an earlier communication (11).

TABLE I

Some properties of the penicillins studied are listed along with the microgram and, where appropriate, bioassay unit equivalents of the test concentrations

Side chain			Salt tested		μmole, μg and bioassay unit equiv. of test concentrations per ml		
Kind		Optical activity	Cation	Mol wt	0.001 μmole	0.010 μmole	0.100 μmole
Benzyl-	G	None	K^+	372.47	0.37 μg 0.59 U	3.73 μg 5.88 U	37.24 μg 58.82 U
Phenoxymethyl-	V	None	K^+	388.47	0.39 μg	3.89 μg	38.85 μg
α-Phenoxyethyl-	B	Levo- or dextro-rotary	K^+	402.49	0.40 μg	4.03 μg	40.25 μg
Aminocarboxybutyl-	N	Levorotary	di-Na^+	403.37	0.40 μg 0.27 U	4.03 μg 2.66 U	40.34 μg 26.62 U

Penicillins. D-,[2] L-,[2] and racemic [3] α-phenoxyethylpenicillins, as well as phenoxymethylpenicillin,[4] were sterilized by exposure of weighed portions of the respective potassium salts to ethylene oxide gas (12) for 18 hours at room temperature. Potassium benzylpenicillin [3] and di-sodium aminocarboxybutylpenicillin [5] were obtained as preweighed sterile, dry powders in ampules. All of these penicillins were dissolved and diluted for testing in tryptic digest of casein-papaic digest of soybean broth.

Stock solutions were prepared which were 0.00100 M, 0.00010 M and 0.00001 M for each of the penicillins studied. Preparation for testing involved transfer of 0.1 ml portions of stock solutions to appropriately labeled series of 13 × 100 mm screw-capped culture tubes before storage at − 22° C. In use, addition of 0.9 ml tryptic digest of casein-papaic digest of soybean broth, containing the inoculum of test staphylococci, resulted in final test concentrations for each penicillin of 0.001, 0.010, and 0.100 μmole per ml. Some characteristics of the penicillins studied and the relationships of micromoles, mass and bioassay units are presented in Table I.

Testing. Test staphylococci from frozen storage were grown out overnight in tryptic digest of casein-papaic digest of soybean broth. According to a direct count in the Petroff-Hausser chamber, a dilution yielding 10,000 staphylococci per 0.9 ml was prepared in sufficient quantity (tryptic digest of casein-papaic digest of soybean broth) to permit inoculation of the requisite 19 tubes from a common supply (3 test concentrations per penicillin; 6 penicillins; 1 control tube containing 0.1 ml sterile broth).

After incubation for 24 hours at 37° C the tubes were inspected. Gross turbidity was accepted as evidence of resistance; absence of visible growth was taken to indicate inhibition. All tubes in which there was inhibition of growth were mixed by swirling, before a 3 mm loopful was removed to inoculate an eighth sector of a tryptic

[2] D-α-phenoxyethylpenicillin, potassium salt, lot 3168–11B4 and L-α-phenoxyethylpenicillin potassium salt, lot L9810, supplied by E. R. Squibb & Sons (Dr. John T. Groel), N. Y.

[3] Racemic α-phenoxyethylpenicillin, (batch 02295) with composition given as 35 per cent D-isomer and 65 per cent L-isomer, supplied by Charles Pfizer & Co. (Mr. Andrew J. Schmitz, Jr.), Brooklyn, N. Y. Potassium benzylpenicillin was supplied through the kindness of Dr. Fredrick L. Fink, also of Charles Pfizer & Co.

[4] Potassium phenoxymethylpenicillin, lot 755838, supplied by Eli Lilly & Co. (Dr. G. E. Maha), Indianapolis, Ind.

[5] Di-sodium aminocarboxybutylpenicillin, supplied as Salmotin test powder, lot 2085-CP, by Abbott Labs. (Dr. J. T. Sylvester), North Chicago, Ill.

TABLE II

Two tellurite-positive staphylococcal isolates were subjected to fivefold repetition of the testing procedure according to which the susceptibility of 200 recent clinical isolates of the tellurite-positive staphylococci to six penicillins was judged *

Isolate no.	μmole ml	Benzylpenicillin					Phenoxymethylpenicillin					α-Phenoxyethylpenicillin L-isomer					α-Phenoxyethylpenicillin D-isomer					α-Phenoxyethylpenicillin DL-mixture					Aminocarboxybutylpenicillin				
163	0.001	R	R	R	S	R	C	C	C	C	C	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
	0.010	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	R	R	R	R	R	R	R	R
	0.100	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	R	S	S	R	R	R	R	R
263	0.001	C	C	S	S	S	C	C	C	C	C	S	S	S	S	S	C	C	S	C	S	C	C	C	C	C	R	R	R	R	R	R	R
	0.010	C	C	C	C	S	C	C	C	C	C	S	S	S	S	S	C	C	C	C	C	C	C	C	C	C	R	R	R	R	R	R	R
	0.100	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	S

* There was concordance of susceptibility within replication units 15 times (of 18 trials) with Isolate 163 and 13 times with Isolate 263. By this assessment, a single determination of susceptibility, by the method described, will give the same result as 5 times repeated testing 88% of the time. R: resistant; S: bacteriostatic; C: bactericidal.

digest of casein-papaic digest of soybean agar plate. Following 24 hours at 37° C in a moist air-candle jar, these agar subcultures were examined. Absence of growth was interpreted as indicating a bactericidal effect, growth as evidence of a bacteriostatic effect in the broth test culture from whence the agar subculture was inoculated. Data supporting such loop subculture differentiation of the nature of the observed inhibitory effect have been presented elsewhere (11).

Assessment of the reproducibility of the results of susceptibility testing by the method described was provided by 5 repetitions of testing with 2 staphylococcal isolates. Each test proceeded from an overnight tryptic digest of casein-papaic digest of soybean broth culture inoculated from the frozen storage culture with counting, dilution, inoculation of penicillin tubes, incubation and interpretation as described. Considering 5 tests at a given concentration of a particular penicillin with one strain of staphylococcus to be a unit of testing, there were 18 units per isolate (Table II). There was agreement in 15 units with Isolate 163; 13 units with Isolate 263. Thus, if testing according to the method described be repeated 5 times, there will be accord in results about 88 per cent of the time; i.e., a single test would suffice 88 per cent of the time. The pattern of disagreements was that of stepwise gradation in effectiveness—variation in effect was between resistant and bacteriostatic, or, bacteriostatic and bactericidal; not resistant and bactericidal.

Penicillinase assay. Gots plates (13) were prepared, using a strain of *Sarcina lutea*[6] which was inhibited in agar pour plates (*Sarcina* inoculum, 1:100 final dilution of a 24 hour broth culture) by 5×10^{-5} μmoles benzylpenicillin per ml. Each staphylococcal isolate was inoculated from an overnight broth culture in the pattern of a small V described on a sixth sector of each of 3 kinds of plates—containing 10^{-4} μmoles per ml of either benzyl-, phenoxymethyl- or DL-α-phenoxyethylpenicillin; all plates having been seeded with *S. lutea* (a 24 hour broth culture in 1:100 final dilution. The plates were incubated at 37° C in moist air-candle jars and inspected at 24 hour intervals for 5 days for appearance of colonies of *S. lutea* juxtaposed to staphylococcal growth.

RESULTS

Since antistaphylococcal effectiveness was observed at three concentrations for each of the six

[6] Kindly supplied by Mr. R. E. Rhodes, Microbiology Section, Smith, Kline & French Labs., Philadelphia, Pa.

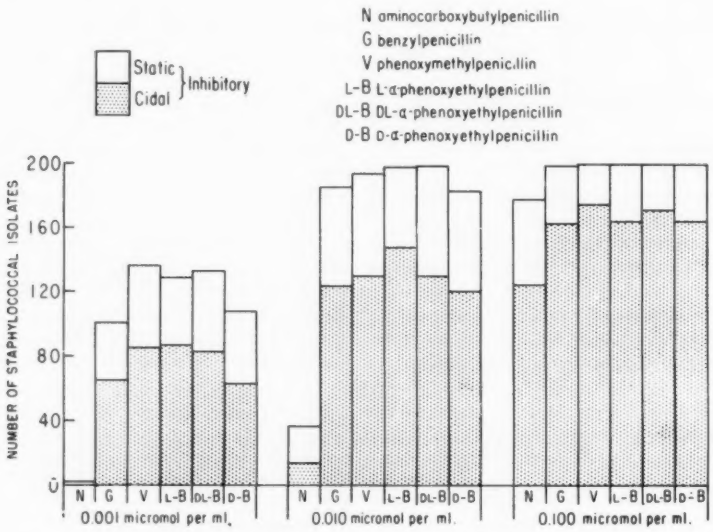

FIG. 2. THE TOTAL HEIGHT OF THE COLUMNS INDICATES THE EXTENT OF INHIBITION OF GROWTH (BACTERIOSTATIC PLUS BACTERICIDAL EFFECT) OF 200 CLINICAL ISOLATES OF TELLURITE-POSITIVE STAPHYLOCOCCI BY 6 PENICILLINS APPLIED AT 3 TEST CONCENTRATIONS. Aminocarboxybutylpenicillin was least potent. Benzylpenicillin and D-α-phenoxyethylpenicillin were about equally effective; both were superior to aminocarboxybutylpenicillin but were less potent than phenoxymethylpenicillin, L-α-phenoxyethylpenicillin or DL-α-phenoxyethylpenicillin; the latter 3 penicillins were nearly identical in potency. Bactericidal potency, the stippled area of each column, varied in the same manner as did inhibition of growth. All of the penicillins tested were more effective when present in high concentrations.

penicillins studied, the results of susceptibility testing are presented (Figure 2) as three groups of data corresponding to the three concentrations employed. It is apparent that with the decimally-stepped increments in penicillin concentration, differences in effectiveness tended to decrease, if not disappear. Thus, aminocarboxybutylpenicillin was virtually impotent at 0.001 μmole per ml, effecting just bacteriostasis with only 2 of 200 isolates. But this same penicillin, when present in a concentration of 0.100 μmole per ml, inhibited the growth of 177 of these same 200 staphylococcal isolates. The other penicillins studied were much more effective than aminocarboxybutylpenicillin, even at 0.001 μmole per ml, but all displayed increased effectiveness at greater concentrations.

Since all six penicillins were effective against most of the test staphylococci at some concentration, differences in effectiveness were quantitative; i.e., potency varied. In order to determine whether or not potency could be related to side chain stereoisomerism, chi square comparison of inhibitory effectiveness (bacteriostatic plus bactericidal effect) and of bactericidal effectiveness was carried out. These results (Figure 3) are best considered one test concentration group at a time.

0.001 μmole per ml. Using phenoxymethylpenicillin, a clinically and pharmacologically well known penicillin as basis for comparison, benzyl-, D-α-phenoxyethyl- and aminocarboxybutylpenicillins were all significantly ($p \leq 0.050$) less effective inhibitors for 200 tellurite-positive staphylococci of clinical origin. Racemic and L-α-phenoxyethylpenicillins were also significantly more active than the D-isomer of penicillin B.

No difference in over-all inhibitory potency distinguished phenoxymethylpenicillin, DL-α-phenoxyethylpenicillin and L-α-phenoxyethylpenicillin. However, detailed comparison revealed that, while 51 of the 200 isolates were resistant, 117 were susceptible to these three penicillins. The remaining 32 isolates were resistant to one or two of the three penicillins, with 4 resistant to phenoxymethylpenicillin alone, 5 resistant only to DL-α-phenoxyethylpenicillin and 6 resistant just to L-α-phenoxyethylpenicillin (Figure 4).

In terms of bactericidal action, the same potency relationships prevailed as were described for inhibition of growth.

0.010 μmole per ml. Both aminocarboxybutylpenicillin and D-α-phenoxyethylpenicillin were again significantly less effective inhibitors of staphylococci than was phenoxymethylpenicillin. At this test concentration, however, benzylpenicillin was not markedly inferior to phenoxymethylpenicillin. Again, DL- and L-α-phenoxyethylpenicillin were significantly more effective than the D-isomer of this penicillin.

Over-all, DL-α-phenoxyethylpenicillin, L-α-phenoxyethylpenicillin and benzylpenicillin were not significantly different from phenoxymethylpenicillin in effectiveness as inhibitors of staphylococci. From isolate-by-isolate comparison, there were 182 of the 200 isolates tested which were susceptible, and 1 which was resistant, to all of these four penicillins. Seventeen were resistant to one or more, but not to all four penicillins. One isolate was resistant to phenoxymethylpenicillin alone, 1 was resistant only to L-α-phenoxyethylpenicillin and 10 were resistant just to benzylpenicillin (Figure 4).

In terms of bactericidal action, phenoxymethylpenicillin was significantly superior to aminocarboxylbutyl penicillin, but was not more active than the other penicillins tested. The pure diastereoisomers and the DL-mixture of α-phenoxyethylpenicillin were not significantly variable in potential for lethal effect.

0.100 μmole per ml. Aminocarboxybutylpenicillin was significantly less effective in securing either inhibition or death of staphylococci than was any of the five other penicillins—all of which were equally effective. Only one isolate was resistant to all six penicillins; another isolate, resistant to benzylpenicillin as well as to aminocarboxybutylpenicillin, was susceptible to the remaining four penicillins tested (Figure 4).

These data suggest the following sequence of antistaphylococcal potency for the penicillins tested:

phenoxymethylpenicillin
L-α-phenoxyethylpenicillin
DL-α-phenoxyethylpenicillin
} > benzyl- > D-α-phenoxyethyl- > aminocarboxybutylpenicillin

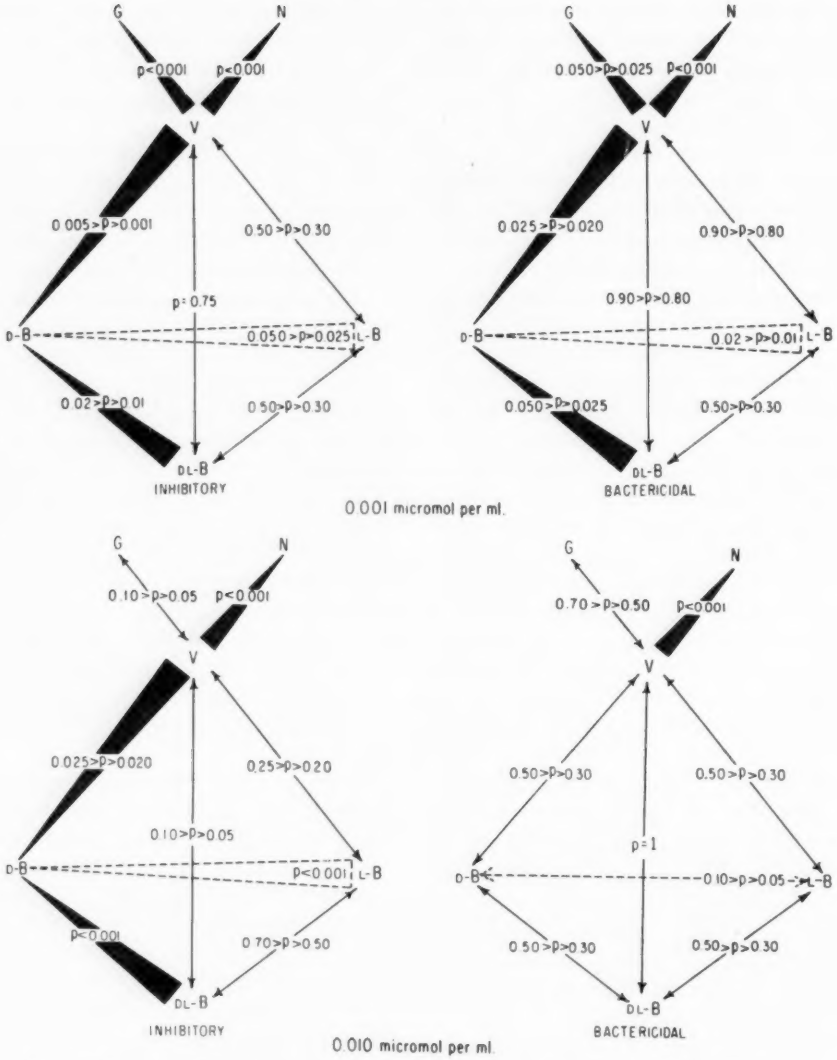

FIG. 3. THE ANTISTAPHYLOCOCCAL POTENCY OF AMINOCARBOXYBUTYLPENICILLIN (N), BENZYLPENICILLIN (G), D-α-PHENOXYETHYLPENICILLIN (D-B), DL-α-PHENOXYETHYLPENICILLIN (DL-B) AND L-α-PHENOXYETHYLPENICILLIN (L-B) WAS EVALUATED BY COMPARISON WITH PHENOXYMETHYLPENICILLIN (V). Two hundred recent clinical isolates of tellurite-positive staphylococci were exposed to all 6 penicillins at the same time, under identical test conditions.

TOP. (0.001 μmole penicillin per ml) These were the relationships whether over-all inhibition of growth or bactericidal effect was considered: penicillin V was significantly ($p \leq 0.050$) more effective than penicillins N, G and D-B; penicillins L-B and DL-B were more effective than D-B; penicillins V, L-B and DL-B were not significantly different.

MIDDLE. (0.010 μmole penicillin per ml) Inhibitory effect: penicillin V was significantly more effective than penicillins N and D-B; penicillins L-B and DL-B were significantly more effective than D-B; penicillins V, L-B and DL-B were not significantly different. Bactericidal effect: penicillin V was significantly more potent than was penicillin N, but was not significantly more often associated with lethal antistaphylococcal effect than were penicillins G, D-B, DL-B or D-B.

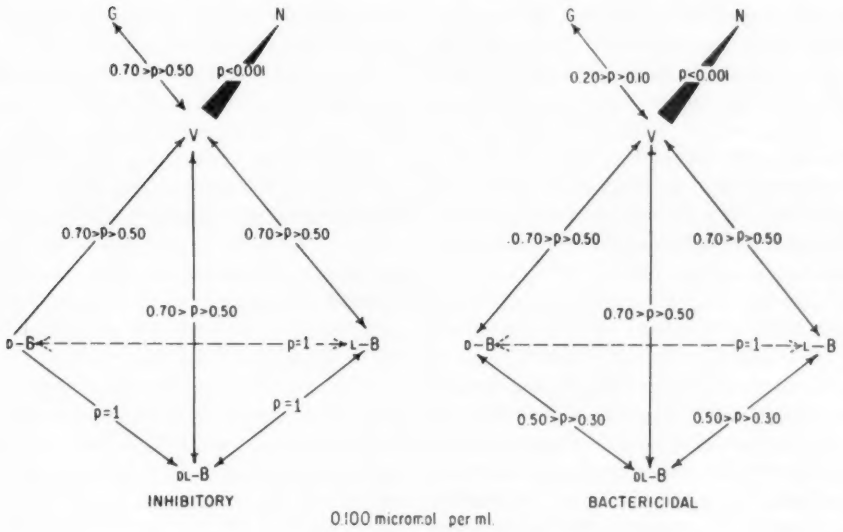

FIG. 3—*Continued*

BOTTOM. (0.100 μmole penicillin per ml) In terms of both inhibitory and bactericidal effect, penicillin N was significantly less effective than penicillins V, G, D-B, DL-B and L-B.

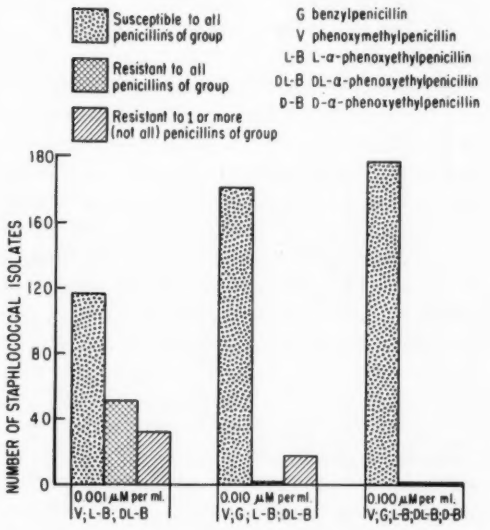

FIG. 4. THE PLOTTED DATA REFER TO ISOLATE-BY-ISOLATE COMPARISON OF THE ANTISTAPHYLOCOCCAL EFFECTIVENESS OF PENICILLIN GROUPS. These groups were delineated at each test concentration by lack of significant variation ($p \leq 0.050$) in over-all inhibitory effect on 200 staphylococcal isolates comparing benzyl-, aminocarboxybutyl-, L-, DL- and D-α-phenoxyethylpenicillins with phenoxymethylpenicillin. As the test concentration increased, the number of penicillins in each group increased and the number of staphylococcal isolates inhibited by all penicillins of a group increased also. Although aminocarboxybutylpenicillin never achieved statistical parity in effectiveness with the other penicillins tested, the major difference between these penicillins in antistaphylococcal effectiveness appears to be quantitative.

Penicillinase activity was first perceptible after 48 hours' incubation of the Gots plates. The *Sarcina* colonies were tiny at this time and were little pigmented. While the total number of positive reactions was greater at 72 than at 48 hours' incubation, there was little increase thereafter. However, observation at 120 hours was considered most reliable, since the deep golden color of the *Sarcina* colonies was then fully developed. Under the test conditions described, penicillinase activity was evident with: 122 isolates against benzylpenicillin; 131 isolates against phenoxymethylpenicillin; 134 isolates against DL-α-phenoxyethylpenicillin. The differences between these three values are not significant ($0.50 > p > 0.30$).

DISCUSSION

Molar description of penicillin concentrations used for susceptibility testing may seem an undue refinement; yet, penicillins are compounds of

known chemical constitution which become irreversibly fixed to bacteria to an extent directly proportional to susceptibility to lethal injury (14). Bound penicillins appear to work their effects by bringing about serious enzymatic dislocations which are only partially understood. Penicillins are antimetabolites and as such are effective as whole molecules. It follows that rational assessment of potency must proceed from description of concentration in molar terms.

In 1957 Sheehan and Henery-Logan (15) reported the laboratory synthesis of phenoxymethylpenicillin. Of little significance in terms of commercial manufacture of penicillins, this chemical milestone was a feat of great significance in permitting demonstration of the essential nature of certain structural features of the bicyclic ring nucleus common to all penicillins. It was known that lysis of the C_7—N_4 bond of the β-lactam ring (see Figure 1) to produce a penicilloic acid, as accomplished by bacterial penicillinases (16), resulted in inactivation of penicillins. From comparison of synthetic with natural penicillins, it became clear that an intact 6-aminopenicillanic acid nucleus was also required in terms of the 5-membered ring and its substituents. More pertinent to the present study was the finding that, for antibacterial activity, all of the optically active centers of 6-aminopenicillanic acid must have the configuration uniformly present in natural penicillins. These observations with 6-aminopenicillanic acid, together with the demonstrated essentiality of particular optical configuration with chloramphenicol (17) and cycloserine (18), are precedent for the affirmation of significant variation in antistaphylococcal effectiveness of penicillins, differing only in optical configuration in the side chain.

Widely publicized in advertisements, the report of Gourevitch, Hunt and Lein (3) indicated that the L-isomer of α-phenoxyethylpenicillin displayed *in vitro* greater antibacterial activity than did the D-isomer against three of five strains of *Staphylococcus aureus* designated: 52-34, 52-75, WR 188, BRL J, BRL O. The superiority of L- over D-α-phenoxyethylpenicillin was attested by respective minimal inhibitory concentrations in micrograms per milliliter of: 1.6 vs 3.1, 3.1 vs 6.2, 3.1 vs 3.1, 0.8 vs 0.8, 0.8 vs 1.6. In the same report, a DL- mixture of α-phenoxyethylpenicillin was: *1*) also more active than D-α-phenoxyethylpenicillin with four of the five test strains of staphylococcus—respective minimal inhibitory concentrations in micrograms per milliliter: 0.8 vs 3.1, 3.1 vs 6.2, 1.6 vs 3.1, 0.8 vs 0.8, 0.8 vs 1.6; *2*) more active than L-α-phenoxyethylpenicillin with strains 52-34 and WR 188—respective minimal inhibitory concentrations in micrograms per milliliter: 0.8 vs 1.6, 1.6 vs 3.1; *3*) equally as active as L-α-phenoxyethylpenicillin with strains 52-75, BRL J and BRL O (3.1, 0.8 and 0.8 μg per ml, respectively).

In addition to indicating that L- and DL-α-phenoxyethylpenicillin were superior in antistaphylococcal effectiveness to D-α-phenoxyethylpenicillin, these meager data were represented as indicating that D- and L-stereoisomers worked in complementary fashion with each other so that a DL-mixture was superior to either single enantiomorph in antistaphylococcal effect. While mouse protection, following on Smith strain staphylococcus infection, was reported to be more successful with

TABLE III

Isolate-by-isolate comparison of the inhibitory effect of the pure diastereoisomers and a mixture (35% D- + 65% L-) of α-phenoxyethylpenicillins on 200 clinical isolates of tellurite-positive staphylococci in a uniform, broth tube-dilution test system *

α-Phenoxyethylpenicillins, μmole/ml	Number of isolates inhibited by					
	D-		L-		DL-	
	Alone	And/or L-, DL-	Alone	And/or D-, DL-	Alone	And/or D-, L-
0.001	1	108	5	129	6	133
0.010	0	182	1	197	1	198
0.100	0	199	0	199	0	199

* Both L- and DL- mixtures were more often solely effective than was the D-isomer. However, since the DL-mixture was not significantly more often alone effective than was the L- form, there is no indication from these data that mixing stereoisomers augments antistaphylococcal potency.

racemic than with either pure diastereoisomer of α-phenoxyethylpenicillin, the authors noted that the differences were not statistically significant.

Determination of the susceptibility of a significant number of clinical isolates of tellurite-positive staphylococci, as described in the present study, indicates that both L- and DL-α-phenoxyethylpenicillins are superior to D-α-phenoxyethylpenicillin.

There was no support from these data for the claim of augmented antistaphylococcal potency on mixing stereoisomers. Over-all, DL-α-phenoxyethylpenicillin was not significantly different in potency from L-α-phenoxyethylpenicillin at any of the concentrations tested (Figure 2). In addition, when isolate-by-isolate comparison of the effectiveness of these three α-phenoxyethylpenicillins was carried out to determine just how often each was the sole inhibitor of a staphylococcal isolate (Table III), no differences were found that were not apparent from the over-all susceptibility data (Figure 2). If complementary effectiveness were gained from mixing D- and L-isomers, the DL- mixture should have been most often the sole effective form of α-phenoxyethylpenicillin. This was not so—these data do not support the notion of augmented potency ascribed to DL- mixture of α-phenoxyethylpenicillin. Yet, in affirming that the L-form was more potent than the D-form, the critical importance of optical configuration is asserted even in the side chain of penicillin. This importance is likely not critical in terms of clinical usage of α-phenoxyethylpenicillin since, generally, overtreatment is clinical practice. However, because chemical variation in acyl radical appears to be the only feasible approach to synthesis of new penicillins, side chain stereoisomerism should be considered in designing new penicillins for synthesis.

Garrod (19) compared *in vitro* the antistaphylococcal activity of benzylpenicillin, phenoxymethylpenicillin and α-phenoxyethylpenicillin (since isomeric designation was not made, it is assumed that a DL- mixture of α-phenoxymethylpenicillin was tested). With 36 "penicillin-sensitive" strains, there were no significant differences in effectiveness of the three penicillins by either plate or tube-dilution testing.

Thirty-eight penicillin-resistant (penicillinase-forming) strains were also tested as part of the same study. When a small inoculum (an 0.02 ml drop of a 1:500 dilution in saline of a broth culture) was applied to an agar culture medium containing penicillin in plate-dilution testing, the three kinds of penicillin were again effective to the same extent. However, by tube-dilution tests in which a large inoculum (an 0.02 ml drop of an undiluted broth culture) was used, both phenoxymethylpenicillin and α-phenoxyethylpenicillin were superior to benzylpenicillin. Moreover, α-phenoxyethylpenicillin was generally more effective than was phenoxymethylpenicillin. As noted by the author, tube-dilution testing with a large inoculum reflects primarily upon the effect of the preformed penicillinase added to the test system as part of the inoculum.

Preparation of inocula in the present study proceeded from broth cultures which by direct count usually had around 10^9 staphylococci per ml; accordingly, dilution to achieve the inoculum, 10,000 staphylococci per 0.9 ml, was at least 100,000-fold. If Garrod's broth cultures for inoculation had attained bacterial populations of density similar to ours—say 5×10^9 per ml—his small inoculum would have delivered about 200,000 and his large inoculum about 250 million staphylococci (as added to 2.5 ml broth, there would have been about 10 million staphylococci per ml). From these considerations, our test situation was more nearly comparable (staphylococci versus penicillins and penicillins versus preformed penicillinase) to Garrod's small inoculum plate-dilution trials than to his large inoculum tube-dilution system. Even so, there remain differences—inoculum size, relative accessibility of penicillins to staphylococci, agar versus broth culture—of such significance that disagreement in results, if not inevitable, is at least not surprising.

Comparison of aminocarboxybutylpenicillin with benzylpenicillin by *in vitro* assay of antistaphylococcal effectiveness, using 30 isolates (20) and 7 isolates (21), indicated that penicillin N was markedly less inhibitory for staphylococci than was penicillin G. The data of the present study, relating to 200 staphylococcal isolates of recent, community-representative origin, support these findings—benzylpenicillin had greater antistaphylococcal potency than had aminocarboxybutylpenicillin. In addition, data were presented which assert that phenoxymethyl- and the

α-phenoxyethylpenicillins are also, individually, more potent antistaphylococcal penicillins than is aminocarboxybutylpenicillin. However, penicillin N is remarkably more active than penicillin G against many species of gram-negative bacilli. Moreover, in addition to being an unusual penicillin in origin from *Cephalosporium* species—a non-*Penicillium* genus of the class *Fungi imperfecti*—the side chain of penicillin N is a hydrophilic, aminoacyl chain which is optically active (8). Unfortunately, we can only speculate on what would be the antistaphylococcal activity of penicillins enantiomorphic and racemic to the tested D-aminocarboxybutylpenicillin.

Application of dense inocula of staphylococci to agar media containing low concentrations of penicillins (about five times the minimal concentration inhibitory for the indicator strain of *S. lutea* which was heavily seeded in the agar media) provided settings designed for demonstration of even meager penicillinase production. On the other hand, the broth susceptibility testing method used exposed small inocula of staphylococci, virtually without preformed penicillinase, to 10-, 100- and 1,000-fold greater concentrations of penicillins than were present in the tests for penicillinase activity. Thus, susceptibility testing and penicillinase assay conditions were sufficiently disparate in design (as in aim) to make reasonable the finding that fewer of the 200 staphylococcal isolates tested were inhibited by even 0.001 μmole of the penicillins per ml than were found to be capable of inactivating these same penicillins. However, assay for penicillinase activity against benzyl-, phenoxymethyl- and DL-α-phenoxyethylpenicillin was carried out to determine whether or not there were differences among these penicillins in terms of susceptibility to staphylococcal penicillinase. Under the conditions of testing employed, there were no significant qualitative differences—results which are in agreement with those reported by McCarthy, Hirsch and Finland (5).

SUMMARY

On the basis of broth tube-dilution susceptibility testing with 200 recent clinical isolates of tellurite-positive staphylococci, the potency of D-, L- and DL-α-phenoxyethylpenicillins was compared with simultaneously and identically tested benzyl-, phenoxymethyl- and aminocarboxybutylpenicillins.

Differences in antistaphylococcal potency were noted which were quantitative: all of the penicillins were more effective when applied in higher concentrations. A potency sequence of the penicillins tested was apparent:

phenoxymethyl-
L-α-phenoxyethyl-
DL-α-phenoxyethyl- } > benzyl- > D-α-phenoxyethyl- > aminocarboxybutylpenicillin

While both L- and DL-α-phenoxyethylpenicillin were superior to D-α-phenoxyethylpenicillin, there was no indication of augmented potency on the part of the DL-mixture in comparison with pure L-α-phenoxyethylpenicillin.

Although side chain optical configuration of α-phenoxyethylpenicillin had significant reflection in antistaphylococcal potency *in vitro*, the major therapeutic implication of this observation lies in the designing of new penicillins for terminal synthesis.

Alpha-phenoxyethylpenicillin, in any of its optical forms, was not a more potent inhibitor of staphylococci than was phenoxymethylpenicillin.

Benzylpenicillin, phenoxymethylpenicillin and DL-α-phenoxyethylpenicillin did not differ qualitatively in susceptibility to inactivation by staphylococcal penicillinase.

Despite the presence of an asymmetric carbon atom in the aminoacyl side chain of aminocarboxybutylpenicillin, the single enantiomorph studied was least effective of the penicillins tested as an inhibitor of staphylococci.

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ADRENAL STEROIDS AND INFECTION: THE EFFECT OF CORTISONE ADMINISTRATION ON POLYMORPHONUCLEAR LEUKOCYTIC FUNCTIONS AND ON SERUM OPSONINS AND BACTERICIDINS *

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Abundant clinical and laboratory experience has established clearly the increased susceptibility to certain infections of animals receiving large doses of cortisone (1–3). There is general agreement that excessively high levels of glucocorticoid hormones diminish the efficiency of host resistance to microbial invasion, but considerable uncertainty remains as to the precise alterations and mechanisms involved (4, 5). Suppression of the inflammatory reaction, impaired function of phagocytic cells, and lowering of antibody levels have all been incriminated to explain the reduced ability to handle microbes of the animal receiving large doses of cortisone.

Host resistance to infections is based ultimately on the activity of many systems: phagocytic cells (polymorphonuclear leukocytes, fixed and wandering macrophages); serum factors (bactericidins, complement, antibody); and the microenvironment of tissue spaces (vascular and neural functions, chemical composition). This communication presents the results of experiments in which the effect of cortisone administration was studied on function of certain of these host resistance systems—namely, the polymorphonuclear leukocyte and serum bactericidins and opsonins.

METHODS

Several experiments were done with different dosages of cortisone. The results were in all cases the same; accordingly, only experiments involving the administration of large doses will be described here.

Young adult New Zealand red rabbits were used. At weekly intervals (× 3) these animals were given an intraperitoneal injection of 300 ml of 0.1 per cent glycogen (Amend Drug and Chemical Co.) in pyrogen-free physiological saline. These injections were intended to prepare the animal for collections of peritoneal exudates at a later date. Before administration of cortisone the rabbits were bled from the heart; after clotting and standing at room temperature for 1 hour, serum was separated by centrifugation and stored in the frozen state for later investigation.

The animals were then given cortisone (Cortone Acetate suspension, Merck Sharp and Dohme) by intramuscular injection on a dosage schedule of 25 mg per kg per day for a total of 11 consecutive days. On Day 11 of cortisone administration blood was again obtained by cardiac puncture and serum prepared and stored as described above.

On Days 10 and 11 of steroid injections, peritoneal exudates were produced and polymorphonuclear leukocytes harvested by a technique described in detail previously (6, 7). In one animal peritoneal exudate leukocytes were also obtained for study 14 days following discontinuation of cortisone injections.

Three different aspects of polymorphonuclear leukocytic function were investigated: *1*) ability to ingest and kill certain microorganisms, *2*) details of cellular morphology before and after ingestion of bacteria, and *3*) content of certain bactericidal agents.

The suspending medium for the tests of phagocytic function was Gey's balanced salt solution containing a final concentration of 0.1 per cent gelatin, hereafter referred to as *gel-Gey's solution*. A portion of the peritoneal exudate was centrifuged (International PR 2) at 1,000 rpm for 5 minutes. The cell button was then washed twice on the centrifuge in 40 ml of gel-Gey's. Exudate cells were in all instances over 95 per cent polymorphonuclear leukocytes. The washed white cells were finally suspended in gel-Gey's solution at a concentration of 20,000 per mm^3. Phagocytic tests were done in 10 × 75 mm Pyrex test tubes into which were placed 0.8 ml of the leukocyte suspension, 0.1 ml of whole or diluted serum, and 0.1 ml of the bacterial inoculum. After capping with sterile rubber stoppers, the tubes were rotated end over end at 38° C. Before incubation and at suitable intervals, samples were removed from each tube and the numbers of surviving bacteria determined by serial dilution in penassay broth and plating on penassay agar. Control tubes containing no white cells always showed a stable bacterial count, indicating that bactericidal effects observed in the test specimens were based on phagocytosis and intracellular killing. The microorganisms employed were *Staphyloccus albus* (Greaves) (nonpigmented, coagulase negative) and *Salmonella typhimurium* RIA, a nonvirulent variant. The mi-

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crobes were cultured overnight on penassay agar slants. The surface growth was suspended in gel-Gey's and washed once on the centrifuge in this medium. Appropriate dilutions were made in gel-Gey's so that the phagocytic test system would contain initially between 10^5 and 10^6 bacteria per ml.

Polymorphonuclear leukocytes from the fresh exudates were examined for morphology and for degranulation following ingestion of foreign material by a procedure described previously (8). In brief, this technique involved sedimentation of granulocytes onto glass slides followed by phagocytosis of bacteria *in situ*. Examination of Wright's stained preparations of these specimens permitted evaluation of various morphological features, and especially of the degranulation response to phagocytosis, in cells obtained from cortisone-treated rabbits.

The remaining exudate leukocytes were collected in the centrifuge, freed from contaminating red blood cells and washed according to the methods described in an earlier report (7). The washed white cell buttons were stored in the frozen state and examined for their content of antimicrobial substances at a later date. This examination was done in parallel with similarly collected and treated white cell buttons from control animals which had not received cortisone. The cell buttons were frozen and thawed 3 times in dry ice-alcohol mixtures and a 38° C bath. The frozen-thawed buttons were suspended in saline at a concentration of 5×10^7 leukocytes per ml, and allowed to stand at room temperature for 30 minutes. These suspensions were then spun at 12,000 G for 15 minutes. The supernatant, called *saline extract*, was stored in the cold. The pellet was washed twice with saline by high speed centrifugation, and the supernatant discarded. The deposit was next suspended in 0.01 M citric acid and, after standing at room temperature for 30 minutes, was centrifuged at 12,000 G. The supernatant of this citric acid extraction was saved for subsequent testing. After two washings with 0.01 M citric acid, the deposit was finally extracted with 0.2 N HCl. This procedure, taken after that described previously (9), permitted separation of three of the major intraleukocytic antimicrobial substances: lysozyme, present in the saline extract; phagocytin, found in the citric acid extract; and histones, located in the hydrochloric acid extract. Assay for lysozyme was performed by the method of Shugar (10) with slight modification. Phagocytin and histone titrations were performed by a technique described previously (9), using *Salmonella* RIA as the test organism.

Comparisons were made of the opsonic activity of serum from normal and cortisone-treated rabbits by employing the phagocytosis system described above. Addition of serial dilutions of serum enabled a titration of opsonic power; i.e., a determination of the lowest concentration of a given serum which manifested a distinct phagocytosis-promoting effect. These observations were made in systems with leukocytes from cortisone-treated animals, as well as in those containing normal rabbit granulocytes.

Measurement of serum bactericidins was done by the technique described in an earlier publication (11), using *Bacillus subtilis* for estimation of β-lysins, and *Escherichia coli* K-12 for titrations of the complement-serum antibody bactericidal system.

RESULTS

General effects of cortisone administration on the experimental rabbits. The rabbits given large doses of cortisone (25 mg per kg per day) for 11 consecutive days lost approximately 10 per cent of their body weight during this period. None showed gross signs of infection or other compli-

TABLE I

Performance in a phagocytic system of polymorphonuclear leukocytes and of serum from normal and cortisone-treated rabbits

Serum, final concentration %		Numbers of surviving bacteria per ml after 60 min at 38° C					
		Normal rabbit leukocytes		Leukocytes from rabbit given cortisone for 11 days		Leukocytes from rabbit 14 days after end of cort. admin.	
		Staph. albus (4×10^5)*	*Sal. RIA* (1×10^6)*	*Staph. albus* (5×10^5)*	*Sal. RIA* (6×10^5)*	*Staph. albus* (6×10^5)*	*Sal. RIA* (8×10^5)*
Normal†	10	9×10^3	3×10^3	2×10^3	8×10^3	3×10^3	7×10^3
Normal	3	6×10^3	2×10^4	2×10^3	2×10^4	6×10^3	2×10^4
Normal	1	6×10^3	6×10^5	1×10^3	3×10^5	5×10^3	6×10^5
Normal	0.3	5×10^4	1×10^6	8×10^3	7×10^5	6×10^4	1×10^6
Cortisone‡	10	8×10^3	4×10^3	1×10^3	6×10^3	1×10^3	1×10^4
Cortisone	3	5×10^3	3×10^4	1×10^3	2×10^4	4×10^3	3×10^5
Cortisone	1	2×10^3	1×10^6	2×10^3	5×10^5	5×10^4	1×10^6
Cortisone	0.3	3×10^4	1×10^6	1×10^4	8×10^5	1×10^5	1×10^6
None		2×10^5	1×10^6	3×10^5	8×10^5	3×10^5	8×10^5

* Bacterial counts at start of incubation.
† From experimental rabbit prior to cortisone administration.
‡ From experimental rabbit after 11 days of cortisone injections.

TABLE II

Performance in a phagocytic system of polymorphonuclear leukocytes and of serum from normal and cortisone-treated rabbits

Polymorphonuclear leukocytes from	Minimal concentration of serum required for >90% phagocytosis and killing in 1 hour at 38° C with			
	Normal rabbit serum		Serum from cortisone-treated rabbit	
	Staph. albus	*Sal. RIA*	*Staph. albus*	*Sal. RIA*
Normal rabbit	1	3	0.3	3
Cortisone-treated rabbit	0.3	3	0.3	3

cating illness before or during cortisone treatment. The sera separated from blood drawn after 10 or 11 days of hormone injections were opalescent, and showed an elevated sugar content (approximately 150 mg per 100 ml) with no detectable ketone bodies, in contrast to the clarity and normal sugar concentration of the pretreatment sera.

Peritoneal exudates, harvested from the rabbits given large doses of cortisone, contained numbers of cells (5×10^8 to 1.5×10^9) similar to those produced in normal animals. Over 95 per cent of these exudate cells in the treated rabbits were polymorphonuclear leukocytes; they differed grossly from normal rabbit granulocyte suspensions only in showing less clumping and less adherence to glass upon standing at room temperature.

Function in phagocytic systems of polymorphonuclear leukocytes and of serum from rabbits treated with cortisone. Comparisons were made *in vitro* of the ability of polymorphonuclear leukocytes from normal and cortisone-treated rabbits to engulf and kill a *Staph. albus* and an avirulent strain of *Sal. typhimurium*. By including in these phagocytic systems dilutions of serum obtained prior to and during cortisone administration, it was also possible to study the effect, if any, of steroid injections on serum opsonic power.

Results of a typical experiment are recorded in Table I, and are also presented in condensed form in Table II. Phagocytic and bactericidal capacity of leukocytes from cortisone-treated animals was the same as that of cells from normal rabbits. Granulocytes obtained 2 weeks after cessation of hormone injections demonstrated no detectable difference in their behavior.

The phagocytosis-promoting power of serum obtained prior to and 11 days after the start of cortisone therapy was not significantly different.

These studies thus demonstrated that large doses of cortisone had no detectable effect on polymorphonuclear leukocytic function or on serum opsonins as measured in this particular phagocytic system.

Studies on morphology and on the degranulation process in polymorphonuclear leukocytes obtained from rabbits receiving large doses of cortisone. Examination of preparations stained by Wright's

TABLE III

Content of antimicrobial agents in granulocytes from normal and cortisone-treated rabbits

Polymorphonuclear leukocyte extracts	Leukocytes from	Content of		
		Lysozyme	Phagocytin	"Histones"
		μg/ml	*U**	*U**
Saline	Normal rabbit	42	160	
	Cortisone-treated rabbit	45	160–320	
Citric acid	Normal rabbit	trace	1,280	
	Cortisone-treated rabbit	trace	1,280	
HCl	Normal rabbit	0		2,560
	Cortisone-treated rabbit	0		2,560

* Reciprocal of highest dilution of the extract which produced greater than a 90% kill of *Salmonella* RIA in 1 hour at 38° C.

TABLE IV

Serum bactericidins in normal and cortisone-treated rabbits

Serum from rabbit	Bactericidal activity* on	
	Bacillus subtilis (β-lysins)	*Escherichia coli* K-12 (complement-antibody)
Prior to cortisone administration	2,000	8–16
After 11 days of cortisone injections	4,000	8

* Reciprocal of highest dilution which produced > 90% kill in 90 min at 38° C.

revealed no significant differences in the appearance of polymorphonuclear leukocytes from peritoneal exudates of normal and of cortisone-treated rabbits. The cells from animals receiving steroid hormone did show some hypersegmentation of nuclei, and perhaps a slight increase in numbers of cytoplasmic granules. The specific granules in these leukocytes showed size, structure and staining properties similar to those of normal rabbit granulocytes.

After ingestion of streptococci or zymozan particles, leukocytes from the cortisone-treated animals showed loss of cytoplasmic granules. This degranulation subsequent to phagocytosis was not quantitatively or qualitatively different from that previously reported for normal rabbit polymorphonuclear white cells (8).

Study of the effect of cortisone administration on the content of antimicrobial substances in polymorphonuclear leukocytes. Polymorphonuclear leukocytes from normal and from cortisone-treated rabbits were extracted in a fashion which permitted estimation of their content of three antimicrobial agents: lysozyme, phagocytin, and histones. Results of these studies, presented in Table III, show that cortisone therapy did not alter the quantity of these bactericidins present in granulocytes.

The lack of demonstrable effect of cortisone therapy on activity of serum bactericidins. Serum samples obtained from the same rabbit prior to and after 11 days of cortisone injections were assayed for their content of two types of bactericidins: β-lysins, and the complement-antibody system. As is seen in Table IV, the level of these serum bactericidal systems was not changed by steroid administration.

DISCUSSION

The increased susceptibility to infections of animals with abnormally high levels of certain adrenal steroids could be due to one of two gross mechanisms: *1*) a direct effect of steroids on the microbes, somehow altering their properties so as to increase their virulence; or *2*) an action on host defense systems resulting in lowered resistance to microbial invasion. Efforts to demonstrate an influence of corticoid hormones on virulence of microorganisms cultured *in vitro* have met with no success; there remains the remote possibility that microorganisms growing *in vivo* might be directly altered by adrenal steroids. At the moment, however, it seems best to assume that native or acquired host resistance mechanisms are disturbed in the presence of high levels of cortisone-like compounds.

The present studies serve to eliminate from consideration several of the host resistance factors which might conceivably be suppressed by elevated corticosteroid concentrations. The morphology, the phagocytic and bactericidal functions, and the content of antimicrobial agents of polymorphonuclear leukocytes were not detectably altered in rabbits injected repeatedly with large doses of cortisone. These results thus support and extend those of Mogabgab and Thomas (12) and of Germuth, Ottinger and Oyama (13). They serve furthermore to cast doubt upon previous work purporting to show diminished phagocytic function of granulocytes from cortisone-treated animals (14, 15).

The mobilization of granulocytes in the cortisone-treated rabbits of the present study was grossly normal; i.e., abundant numbers of these cells were obtained in exudates produced by intraperitoneal injection of glycogen. However, the migration of granulocytes into tissues of these animals in response to implantation of small numbers of certain microbes might well have been deficient or significantly delayed, in keeping with the findings of several previous studies on this point (reviewed in Reference 5).

Investigations into relationships between serum antibodies and cortisone administration (4, 5) have yielded somewhat conflicting results, but there seems now to be a measure of agreement that suppression or elimination of antibodies is not primarily responsible for the altered host resistance. The present observations support this view, and they also demonstrate that serum from cortisone-treated rabbits possesses a normal concentration of heat-labile opsonins. The lack of influence of cortisone therapy on the level of serum bactericidins for coliform microbes provides further evidence that high levels of glucocorticoids do not significantly suppress antibodies or complement, since these components are both essential for this bactericidal activity.

The present experiments thus indicate that the depression of host resistance to infection which occurs in association with high levels of corticosteroid hormones is not based upon alterations in polymorphonuclear leukocytic functions or in serum opsonins and bactericidins. Studies done by others (reviewed in References 4 and 5) indicate furthermore that changed behavior of mononuclear phagocytes is not primarily responsible for the cortisone effect on susceptibility to microbial disease. Thus, the evidence available at present suggests that adrenal glucocorticoids depress host resistance by suppressing the inflammatory response, and perhaps also by altering metabolism so as to render the local tissue chemical environment more favorable for proliferation of various microbes.

SUMMARY

Polymorphonuclear leukocytes collected from rabbits given large doses of cortisone exhibit normal capacity to engulf and kill certain staphylococci and enteric bacteria. The general morphology and degranulation response to phagocytosis of these cells are not detectably different from those of leukocytes from normal rabbits.

Granulocytes from normal and from cortisone-treated rabbits contain similar amounts of the antimicrobial agents lysozyme, phagocytin and histone.

Administration of large doses of cortisone to rabbits is not associated with significant changes in opsonic or bactericidal activities of the serum.

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COMPARATIVE BINDING OF BEEF AND HUMAN INSULIN TO INSULIN ANTIBODIES PRODUCED IN MAN AND GUINEA PIGS*

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Two methods (1, 2) for the immunochemical assay of circulating human insulin have been described recently. Both are based on the displacement of insulin-I^{131} from antibody by the addition of unlabeled insulin but differ in the conditions employed for the combination of insulin to antibody and for the subsequent separation of the bound from free insulin. Beef insulin was used as the reference standard by Grodsky and Forsham (1) because *a*) human insulin of sufficient purity to allow determination of its specific insulin content by biological assay was not available, and *b*) it was shown that human and beef insulins bind to guinea pig antibeef insulin antibody in a proportional relationship. Yalow and Berson (2) criticized the use of beef insulin as a standard, stating that *human* and *beef* insulins react disproportionately with guinea pig antiserum.

In other communications (3, 4), a 25- to 100-fold difference between the affinity of human insulin and beef insulin for antibodies produced in the human against antibeef-pork insulin was reported.

The importance of these phenomena in the immunoassay of circulating insulin, as well as their effect on the metabolism of this hormone, has recently become apparent. On the basis of these considerations, we undertook to reinvestigate the quantitative relationship between beef and human insulin for binding to guinea pig and human antibody.

MATERIALS AND METHODS

Bovine crystalline insulin, lot no. T-2842, was obtained from Eli Lilly & Co. Fisher human insulin[1] was received in 1960 from the Connaught Labs., Toronto, Canada (assay in 1956 = 6.8 U per mg based on a standard containing 22 U per mg). Individual extracts containing crude human insulin were prepared from human pancreas obtained at surgical biopsy (preparation A) or at autopsy (preparations B and C) by a modification (5) of the Jephcott procedure (6). An identical extract of bovine insulin was similarly prepared from fresh ox pancreas. Human antiserum was obtained from a subject with insulin-resistant diabetes at least 24 hours after any injection of beef-pork insulin.

Comparative effect of changing concentration of beef or human insulin on the binding of beef insulin-I^{131} to guinea pig insulin antibodies. The binding of beef insulin-I^{131} to antibodies against beef insulin produced in guinea pigs, and the subsequent precipitation of the bound insulin with sodium sulfite was described in a previous report (1). In some of the later studies precipitation was accomplished with sodium sulfate at 25° in place of sodium sulfite at 4° C. Sodium sulfate produced a more compact, easily centrifuged precipitate; however, results in either case were essentially the same. Each of the human insulin preparations was initially diluted with 5 per cent albumin so that after incubation and salt precipitation, the per cent insulin-I^{131} activity displaced into the supernatant was comparable with that produced by a beef insulin standard of 150 μU of insulin per ml. Thereafter, progressively increasing or decreasing concentrations of the diluted human insulin preparations were similarly treated, and the resulting per cent activity in such supernatant was plotted. If changing human insulin concentration causes a displacement of insulin-I^{131} from antibody proportional to that effected by changing beef insulin concentration, the curves obtained for the human and the beef insulins should coincide. This technique eliminates the necessity for using calculations of insulin content of the crude human preparations based on a biological assay.

The comparative affinity of human and beef insulin for human antibeef-pork insulin antibodies. Each preparation of human insulin was assayed by incubation with *guinea pig* antiserum and by comparison with a reference curve made after incubation of beef insulin with the same *guinea pig* antiserum.[2] The human insulin preparations were then reassayed by incubation with *human*

* Supported by Grant A-3869 from the National Institute of Arthritis and Metabolic Diseases and by a grant from Eli Lilly & Co.

[1] We are greatly indebted to Dr. A. M. Fisher for supplies of this preparation.

[2] Two different dilutions of the human insulin preparations were used for each determination. Determinations were repeated on 3 to 18 different days.

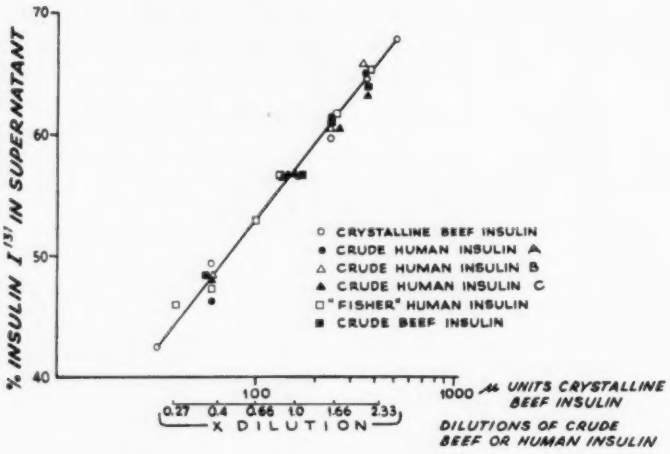

FIG. 1. COMPARISON OF THE EFFECTIVENESS OF PROPORTIONAL DILUTIONS OF VARIOUS INSULIN PREPARATIONS IN DISPLACING BEEF INSULIN-I^{131} FROM GUINEA PIG ANTI-INSULIN SERUM.

antiserum and comparison with a reference curve made after incubation of beef insulin with *human* antiserum. If the affinity of human insulin for human antibody is poor, the assay of the human insulin in systems using human antisera would be much lower than the comparative assay against guinea pig antisera. In this study, too, a biological assay of the human preparations was not necessary.

RESULTS

For the range of insulin concentrations employed, the percentage of radioactivity in the supernatant after incubation was generally proportional to the log of the concentration of added unlabeled insulin. The supernatant activities after incubation of various dilutions of each of the human insulin preparations are plotted in Figure 1. The three crude human insulin preparations, the Fisher insulin, and the extract of beef pancreas produced points which, within the error of the method, coincided exactly with the reference curve produced by the crystalline beef insulin. The Fisher insulin, when assayed against guinea pig antibeef insulin antibody, contained 4.04 ± 0.87 U or 144 ± 31 μg of insulin per mg ($\pm = 2 \times$ SE). Results were unchanged when the incubations were carried out at pH 7.4 instead of 8.6 or for periods of 4 days instead of 1 hour.

Results of assays using crystalline beef insulin as reference standards for the crude human insulin preparations A and C, Fisher human insulin, and the crude beef insulin either were not significantly different or varied less than one-fold regardless of whether *human antiserum* or *guinea pig antiserum* was employed in the incubations (Figure 2). Although the assay of human insulin preparation B was significantly lower ($p = <$ 0.001) when measured against human antisera than when measured against guinea pig antisera, the quantitative difference was less than threefold.

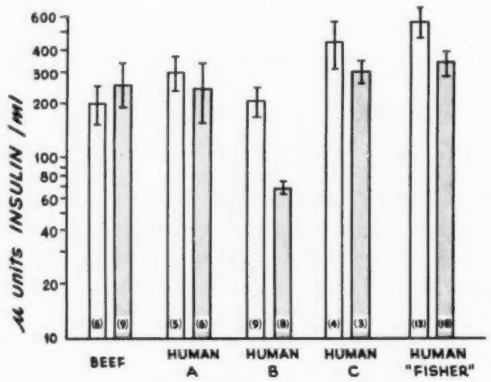

FIG. 2. IMMUNOCHEMICAL ASSAY OF BEEF AND HUMAN INSULIN IN SYSTEMS EMPLOYING INSULIN ANTIBODIES PRODUCED IN MAN AND GUINEA PIGS. Crystalline beef insulin was used for all reference curves. Figures in parentheses equal total number of determinations; $I = 2 \times$ SE. Plain columns: unknowns and reference standards compared against guinea pig antiserum. Dotted columns: unknowns and reference standards compared against human antiserum.

DISCUSSION

Our previous finding (1) that different concentrations of human and of beef insulin produce proportionate changes in the binding of insulin-I^{131} to guinea pig antibody has been confirmed with four different preparations of human insulin. Therefore, under the experimental conditions employed, changing concentrations of human insulin may be equated with an immunochemical system employing beef insulin standards.

According to Yalow and Berson (2), "beef insulin cannot be used as a standard for the assay of human insulin in the guinea pig antibody insulin system" and investigations employing beef standards are to be considered "invalid." The conditions of their experiments (pH 7.4, incubation time 4 days), however, were quite different from those used by Grodsky and Forsham (1) (pH 8.6, incubation time 1 hour). Since both pH (7) and time of incubation (8) have a known effect on formation of insulin-antibody complexes, it was initially believed that differences in these or less obvious experimental conditions could have accounted for the different conclusions reached by the two investigations. In our experiments neither incubation at pH 7.4 nor incubation for 4 days at 4° C altered the results. Although Yalow and Berson noted that "the relative degree to which human insulin and beef insulin react at different insulin concentrations varies severalfold," these conclusions are difficult to reconcile with their own data, as shown in Figure 3. If the ratio of bound to free insulin-I^{131} (B/F) obtained after incubation with the concentrations of Fisher human insulin estimated at 0.25, 0.5, 1.0, 2.0, 2.5 and 3.0 mμg per ml are read against the crystalline beef insulin curve, the resultant assays in "beef insulin equivalents" are 0.12, 0.27, 0.55, 1.0, 1.12 and 1.35 mμg per ml, respectively. The proportionality (beef insulin equivalents/ assumed insulin content) varied only from 0.45 to 0.55 *regardless of the point chosen along the curves for the calculations.* Therefore, although the curves of Yalow and Berson for human and beef insulin diverge, *they remain proportional to each other* throughout the range where B/F is sensitive to changing insulin concentration. Fisher human insulin was biologically assayed at 6.8 U per mg in 1956, "but it was believed that the activity of the latter preparation might have decreased slightly . . ." (2). These authors assigned a value of 6.0 U or 282 μg [3] per mg to the Fisher insulin but did not report the calculations employed for estimating the insulin degradation over a 3 year interval. If one takes the actual insulin content of this preparation to be 0.45 to 0.55 of the estimated 6.0 U and corrects for the difference in unit size between the Toronto standard (22 U per mg) and the Eli Lilly & Co. standard (27 to 29 U per

[3] This value representing $6/22 \times 1{,}000$ is more correctly 273 μg.

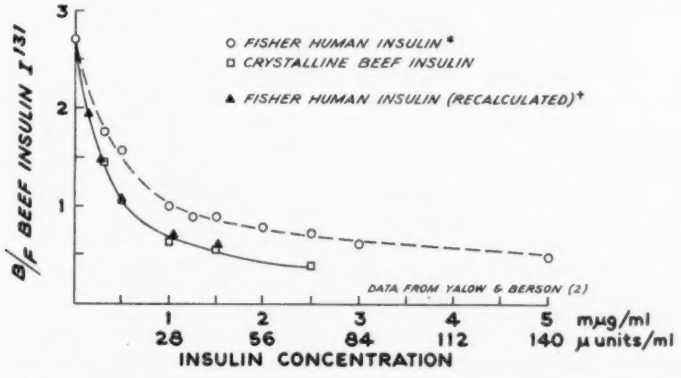

FIG. 3. COMPARISON OF THE EFFECTIVENESS OF VARIOUS CONCENTRATIONS OF FISHER HUMAN INSULIN AND CRYSTALLINE BEEF INSULIN IN REDUCING THE BOUND TO FREE (B/F) RATIO FOR BEEF INSULIN-I^{131}. * Data plotted on basis of insulin content of Fisher preparation estimated as 282 μg per mg (2). † Same data plotted on basis of insulin content of Fisher preparation assayed by Eli Lilly & Co. as 146 μg per mg.

mg), a value of 3.44 to 4.21 U or 122.5 to 150 μg of insulin per mg for Fisher insulin is obtained. This value is in excellent agreement with our own finding of 4.04 ± 0.87 U or 144 ± 31 μg of insulin per mg, based on immunoassay against a crystalline beef insulin standard. A portion of the Fisher insulin was sent to Eli Lilly & Co. as a blind sample for biological assay. Its potency was reported to be 4.08 ± 19.8 per cent U per mg[4] ($p = 0.05$). If one replots the data reported by Yalow and Berson using this estimate of insulin content, the curves for human and beef insulin are almost superimposable (Figure 3). On the basis of our findings and the data of Yalow and Berson, it therefore appears that human and beef insulin effectively cross react in a proportional and nearly equivalent manner. [As stated previously, however, the exact evaluation of the quantitative relationship between human and beef insulin must await the availability of a crystalline insulin of sufficient purity to allow unequivocal determination of its insulin content by biological assay (1).] Since the Fisher preparation was used as a standard by Yalow and Berson for their assays of circulating insulin in human sera, their values perhaps should be reduced by approximately 50 per cent. Thus, normal human circulating insulin estimated at 27 μU per ml (2) may be closer to 13 μU per ml.

In the report that the affinity of human insulin for human antibody was 25 to 100 times less than that of beef insulin, it was suggested that the nature of the three amino acids under the disulfide bridge in human insulin might be responsible for such differences in antigenicity (4). In contrast, our results showing the comparative agreement between the assay values for human insulin against beef insulin standards, when either guinea pig or human antisera were used, suggest that *extracted* human insulin binds almost as well to human anti-beef-pork insulin antisera as does beef insulin. The finding by Nicol and Smith (9) that human insulin and pork insulin have an identical amino acid structure under the disulfide bridge strongly indicates that antigenic differences between extracted human insulin and insulin from this species (if they exist) do not occur at that site on the insulin molecule.

[4] We are indebted to Mr. William Baum for the biological assay of this preparation.

SUMMARY

The comparative effect of changing the concentration of beef or human insulin on the binding of beef insulin-I^{131} to insulin antibodies produced in the guinea pig or man was reinvestigated, using a technique involving the separation of bound from free insulin by preferential salt precipitation.

Varying dilutions of four different preparations of crude human insulin produced changes in the binding of insulin-I^{131} to guinea pig antibody proportional to changes in concentration of crystalline beef insulin. *Biological assay* of the purest human insulin (Fisher insulin) was 4.08 ± 19.8 per cent U per mg. *Immunological assay*, using beef insulin as reference standard was 4.04 U per mg, SE = 0.43. It was concluded that *extracted* human and beef insulin cross react with guinea pig antibeef insulin in a proportional and nearly equivalent manner.

Agreement between the immunoassay values for human insulin against beef insulin standards when either guinea pig or human antisera were used was demonstrable, suggesting that *extracted* human insulin binds almost as well to human anti-beef-pork insulin antisera as does beef insulin.

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STUDIES ON THE ACTION OF HAGEMAN FACTOR: EVIDENCE THAT ACTIVATED HAGEMAN FACTOR IN TURN ACTIVATES PLASMA THROMBOPLASTIN ANTECEDENT *

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Hageman factor is a component of normal plasma which participates in the early stages of blood coagulation (1). In freshly drawn blood, Hageman factor seems to be inactive, but contact with glass or certain other adsorbents changes it to an active form (2–8). The specific function of this activated Hageman factor has been puzzling. Several investigators have proposed that it reacts with a second clotting factor—plasma thromboplastin antecedent—to produce a clot-promoting substance (5, 9–12). In turn, the function of this clot-promoting substance remains to be explained, although its interaction with Christmas factor has been suspected (11, 13).

In the present study, techniques are described for preparing Hageman factor and plasma thromboplastin antecedent, each apparently deficient in other recognized clotting factors. The availability of these fractions has made it possible to examine their interaction. Evidence will be presented that activated Hageman factor converts plasma thromboplastin antecedent into a clot-promoting substance—activated plasma thromboplastin antecedent—by an enzymatic process. In turn, the clot-promoting substance probably acts enzymatically, although its substrate is not yet defined.

NOMENCLATURE

The terminology used to describe the clotting factors and hemorrhagic disorders has been summarized elsewhere (14). Briefly, *Hageman trait* refers to a familial disorder of the early stages of coagulation, first observed in a man without hemorrhagic symptoms (1). The plasma of patients with Hageman trait behaves as if it were deficient in *Hageman factor* (1). *Plasma thromboplastin antecedent* (PTA) deficiency is a familial hemorrhagic disease in which the plasma appears to be deficient in PTA (15). *Antihemophilic factor* and *Christmas factor* are deficient in the plasma of patients with classic hemophilia and Christmas disease [or plasma thromboplastin component (PTC) deficiency], respectively.

The initial stages of the coagulation of plasma *in vitro* involve the interreactions of Hageman factor, PTA, Christmas factor and antihemophilic factor to form plasma thromboplastin. Platelets or certain phospholipids and calcium ions are also intimately concerned with the formation of *plasma thromboplastin*. In turn, plasma thromboplastin, in some undefined association with *Stuart factor* (Factor X), *proaccelerin* (Factor V), and *calcium ions*, converts *prothrombin* to *thrombin*. Thromboplastin may also be derived from tissues; *tissue thromboplastin* is optimally effective only in the presence of the precursor of serum prothrombin conversion accelerator (*pro-SPCA* or Factor VII). These interrelationships are demonstrated in Figure 1.

MATERIALS AND METHODS

Unless otherwise noted, all studies were performed with blood, plasma or plasma fractions which had been collected or prepared in silicone-coated tubes and had never wittingly been in contact with glass or similar clot-promoting surfaces.

Venous blood was drawn from the antecubital veins of normal subjects or from patients with various coagulative disorders, using no. 18 gauge needles coated with Arquad-2C (Monocote-E, Armour) and glass syringes coated with silicone (G.E. Dri-Film SC-87).

Experiments were performed with blood obtained from 5 patients with Hageman trait and 3 with PTA deficiency. The five with Hageman trait included J.H., M.R. and T.R., who were Patients I, II and III in an earlier publication (1); V.S., a 35 year old woman; and T.W., a 12 year old girl. V.S. and T.W. each has a brother with the same defect. Only V.S. has had symptoms suggesting a bleeding tendency; she had vaginal bleeding during the early months of a pregnancy still in progress. The patients with PTA deficiency were M.W. and B.Y., two of the three patients described in Rosenthal, Dreskin and Rosenthal's original study (15), and G.P., whose case was reported by Todd, Panter and

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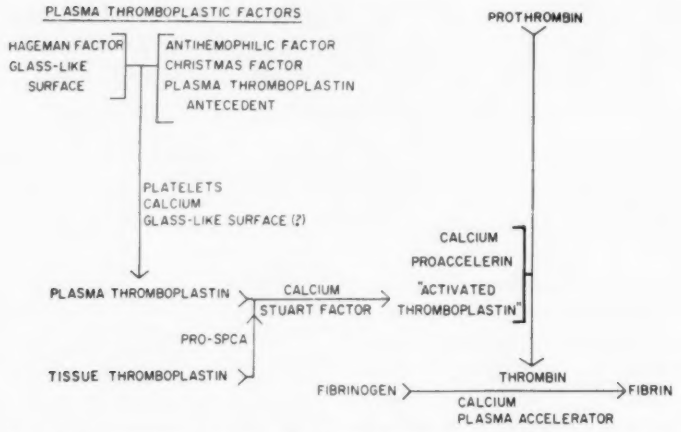

FIG. 1. A TENTATIVE OUTLINE OF SOME STEPS IN THE CLOTTING PROCESS. Reprinted with permission of C. C Thomas, Springfield, Ill. (14).

Wright (16). M.W. and B.Y. were bled in New York and their frozen plasma carried to Cleveland, while G.P. was bled at different times in New York or Cleveland. The identity of the defect in the three patients with PTA deficiency has been confirmed by both Dr. Robert Rosenthal (17) and ourselves.

Platelet-deficient citrated plasma was prepared by an adaptation of the method of Conley, Hartman and Morse (18). Thirty ml of venous blood was withdrawn and discarded. Next, 30 ml or more of venous blood was drawn through the same needle with fresh syringes. This blood was mixed with one-ninth its volume of 0.13 M trisodium citrate and its cells were removed by centrifugation in silicone-coated Lusteroid tubes at 2,700 G for 15 minutes in an International SB size 1 centrifuge. The plasma was recentrifuged twice for 10 and 30 minutes, respectively, at 27,000 G in an International high speed centrifuge. This platelet-deficient plasma was kept in silicone-coated Lusteroid tubes. It was then refrigerated in ice water and used within a few hours or stored at $-25°$ C until used. *Unless otherwise noted, this platelet-deficient, citrated plasma, which had had no contact with glass was used in all the experiments to be described.*

Aluminum hydroxide-adsorbed plasma is deficient in prothrombin, pro-SPCA, Stuart factor and Christmas factor. It was prepared by incubating 10 vol of plasma with 1 vol of aluminum hydroxide gel for 3 minutes at 37° C in silicone-coated Lusteroid tubes. The mixture was then centrifuged for 5 minutes at 2,700 G. The supernatant plasma was usually used immediately, but its content of inactive Hageman factor and PTA was preserved by storage at $-25°$ C for several months or more. For different purposes, dilute or concentrated aluminum hydroxide gels were used.

Dilute aluminum hydroxide gel was prepared by diluting 1 part of a commercial suspension [1] with 3 parts of water, while *concentrated aluminum hydroxide gel* was undiluted Amphojel.[2] The concentration of aluminum hydroxide gels, as provided by the manufacturers, is expressed in terms of aluminum oxide. The dilute aluminum hydroxide gel contained 0.6 per cent and the concentrated gel 4 per cent aluminum oxide; the latter also contained small, unspecified quantities of glycerin and sodium benzoate.

Barium sulfate-adsorbed serum is deficient in prothrombin, pro-SPCA, Stuart factor and Christmas factor. It was prepared by incubating venous blood for 4 hours at 37° C in Pyrex tubes. The serum was then separated by centrifugation, incubated for 20 hours at 37° C and stored at $-25°$ C. Prior to use the serum was adsorbed for 10 minutes at room temperature with 100 mg of powdered barium sulfate (Baker) per ml. After centrifugation the adsorbed serum was used within a few hours.

Heated plasma was prepared by incubation in a water bath. The temperature was determined by immersing the bulb of a silicone-coated thermometer in the tube of plasma or was estimated by immersing a thermometer in a similar tube containing water and heated simultaneously in the same section of the water bath.

Ammonium sulfate fractionation of plasma was performed in silicone-coated Lusteroid tubes by the addition of ammonium sulfate solution, saturated at 4° C and neutralized by the addition of 10 ml of concentrated ammonium hydroxide per L. The mixture was stored at 4° C for 1 hour or more and centrifuged for 5 minutes at 2,700 G. Each fraction was reprecipitated a second time prior to testing. Each was then dissolved in a volume of buffer equal to that of the original plasma and dialyzed against buffer for 48 hours.

Carboxymethylcellulose-treated plasma was prepared by shaking 1 ml of plasma with 75 mg of carboxymethyl-

[1] Provided through the courtesy of Cutter Labs., Berkeley, Calif.

[2] Without flavor, furnished through the kindness of Wyeth Labs., Philadelphia, Pa.

cellulose for 10 minutes in uncoated Lusteroid tubes (ID 11 mm) in a mechanical shaker. The carboxymethylcellulose was then removed by centrifugation at 2,700 G for 5 minutes. Carboxymethylcellulose, containing approximately 0.72 mEq of acidic groups per g of cellulose, was prepared by the procedure of Ellis and Simpson (19).

Crude Hageman factor, deficient in PTA, was prepared from plasma of patients with PTA deficiency. The plasma was adsorbed with dilute aluminum hydroxide gel to remove prothrombin, pro-SPCA, Stuart factor and Christmas factor; this treatment did not decrease the titer of Hageman factor appreciably. The plasma was then heated at 56° C for 0.5 hour; this process decreased the titer of antihemophilic factor and proaccelerin, and precipitated fibrinogen but did not usually decrease the titer of Hageman factor. However, some preparations lost as much as half or more of their activity after adsorption and heating.

The adsorbed, heated plasma was then diluted to twice its volume by the addition of 0.15 M sodium acetate (pH 5.2) and sufficient 1 M acetic acid to bring the plasma to pH 5.2. The acidified plasma was diluted with an equal volume of water so that it was now at 4 times the original plasma volume, ionic strength 0.075 and pH 5.2. It was next stirred at room temperature for 10 minutes with 75 mg of carboxymethylcellulose for each ml of the original plasma, and centrifuged for 5 minutes at 2,700 G. The carboxymethylcellulose was washed twice with 0.075 M sodium acetate buffer (pH 5.2) and eluted twice at room temperature for 5 minutes each time with a mixture of equal amounts of 0.067 M sodium phosphate buffer (pH 6.8) and 1 M sodium chloride. Each elution was done with a volume of eluting solution one-half that of the original plasma. The two eluates were combined, recentrifuged to remove traces of carboxymethylcellulose, and dialyzed for 16 to 24 hours against buffer. The crude Hageman factor was stored in silicone-coated Lusteroid tubes at − 25° C until used.

Crude Hageman factor, prepared in this way, contained 1 per cent or less of the protein of the plasma from which it had been prepared. In most preparations it retained substantially all of the plasma's capacity to correct the abnormality in plasma deficient in Hageman factor. This crude Hageman factor was used in the experiments to be described. Further purification was obtained by column chromatography, adsorbing crude Hageman factor onto calcium phosphate gel and eluting it with a gradient of equal parts of 0.05 M and 0.5 M sodium phosphate buffer at pH 6.8. Still further separation from other proteins could then be made by using phosphocellulose. Details of this purification will be published at a later date; preliminary data indicate a minimal purification of about 1,000-fold, compared with the plasma from which it was prepared, although the significance of such comparisons is made difficult by the nature of the assay system.

Crude plasma thromboplastin antecedent, deficient in Hageman factor, was prepared in the same way as crude Hageman factor from the plasma of patients with Hageman trait. The plasma was adsorbed with dilute aluminum hydroxide gel to remove prothrombin, pro-SPCA, Stuart factor and Christmas factor. This adsorption usually resulted in a decrease in the plasma's PTA activity of 75 to 80 per cent. The adsorbed plasma was then heated at 56° C for 30 minutes to precipitate fibrinogen and to decrease the titer of antihemophilic factor and proaccelerin. Plasma heated at 56° C had only about one-fourth or less of its original ability to shorten the clotting time of PTA-deficient plasma. However, when plasma was successively adsorbed with dilute aluminum hydroxide gel and heated at 56° C for 30 minutes, the loss of PTA activity was no greater than when either procedure was performed separately, so that adsorbed, heated plasma retained one-eight to one-fourth of its corrective capacity for PTA-deficient plasma.

The adsorbed, heated plasma was then adsorbed with carboxymethylcellulose, and crude PTA was eluted in the same manner as Hageman factor. This fraction contained about 1 per cent of the protein of the untreated plasma, but because of the losses in the initial steps of preparation represented only a 10- to 20-fold purification of activity; again, the nature of the assay makes an estimate of the degree of purification hazardous. Additional purification of this crude PTA could not be achieved, for reasons to be discussed in Section VII of Results.

A fraction of plasma that inhibits active Hageman factor and active plasma thromboplastin antecedent was separated from pooled outdated citrated plasma obtained from the blood bank. The plasma was adsorbed with concentrated aluminum hydroxide gel and heated at 56° C for 30 minutes. The fraction of plasma soluble at 50 per cent saturation but insoluble at 70 per cent saturation with ammonium sulfate was separated by centrifugation for 5 minutes at 27,000 G. This fraction was dissolved in buffer, reprecipitated at 70 per cent saturation, and collected on Whatman no. 54 filter paper on a Buchner funnel. The precipitate was dissolved in 1/6 vol of water, dialyzed overnight against 0.01 M sodium acetate (pH 5.2) and the precipitate discarded after centrifugation. The supernatant solution was dialyzed against barbital-saline buffer; it contained the inhibitory activity, about fourfold concentrated as compared with the original plasma. Its degree of purification was not determined. In this state, the inhibitory property was stable for several months at − 25° C; this preparation was used in the experiments described. Further purification was possible by precipitating the fraction soluble at 22.5 per cent saturation with ethyl alcohol at pH 7.5 and ionic strength 0.063, but insoluble at pH 5.6. However, this fraction was too unstable for further study. The inactivating property is also present in plasma fractions IV-1 and IV-4, as separated by Cohn Method 6.[3]

Diisopropyl fluorophosphate (DFP)-treated plasma or plasma fractions were prepared by incubation of the appropriate plasma and DFP (K. & K. Labs., Long Island City, N. Y.) in barbital buffer in dialysis tubing at

[3] Ratnoff, O. D. Unpublished observations.

25° C for 1 hour, followed by dialysis against several changes of buffer at 4°. In some cases, the pure DFP was initially diluted in anhydrous isopropanol before the final dilution was made. As a control for the latter experiments, pure isopropanol was added to a similar plasma preparation to ascertain whether any of the inhibition observed might be due to the isopropanol. In no case did the amount of isopropanol used produce any inhibition.

Concentrations of Hageman factor, plasma thromboplastin antecedent, antihemophilic factor and Christmas factor were assayed by measuring the effect of the sample to be tested upon the recalcified clotting time of plasma obtained from individuals with congenital deficiencies of these factors (20, 21). *Concentrations of prothrombin* (22), *proaccelerin* (21), *pro-SPCA* (21) and *Stuart factor*[4] (23) were estimated by published methods. To test for *thrombin*, 0.1 ml of the sample was incubated with 0.1 ml of bovine fibrinogen solution at 37° C overnight. In addition, the same mixture was incubated in the presence of 0.1 ml of 0.025 M calcium chloride, since this salt potentiates the action of thrombin. The solution of *bovine fibrinogen*, prepared commercially, was said to contain 300 mg of coagulable protein per 100 ml.[5]

The *thrombin time* (1) and *prothrombin time* (24) were measured by previously described techniques. Commercial acetone-dried rabbit brain (Difco) was used as a source of *tissue thromboplastin*. To test the effect of plasma fractions upon *serum prothrombic activity*, 0.1 or 0.2 ml was pipetted into new Pyrex tubes. Sufficient venous blood was added to bring the volume to 2.25 ml, and each tube was then inverted twice over Parafilm (Marathon). The tubes were incubated for suitable periods at 37° C, stirred vigorously with 0.25 ml of 0.13 M sodium citrate, and re-incubated for 30 minutes. The tubes were then centrifuged at room temperature for 15 minutes at 1,900 G, and the prothrombin content of the serum was measured by the method of Alexander (22). The prothrombin content was compared with that of samples of plasma of the same blood obtained from similar tubes to which 0.25 ml of 0.13 M sodium citrate was added before the addition of blood.

"Cephalin"-fortified clotting time, an adaptation of the partial thromboplastin time (25), was devised to test the effect of clot-promoting fractions upon the clotting time of normal or abnormal plasmas in silicone-coated tubes. Duplicate determinations demonstrated the unreliability of the recalcified clotting time in silicone-coated tubes unless coagulation was accelerated by the addition of traces of "cephalin." The test was performed by incubating 0.1 or 0.2 ml of the fraction to be tested, 0.1 ml of plasma and 0.1 ml of a "cephalin"-calcium mixture in silicone-coated tubes at 37° C. The tubes were tilted once a minute until clotting occurred. Duplicate determinations usually agreed within 1 minute when the clotting time was 20 minutes or less and within 3 minutes when the clotting time was 30 minutes or less. The *"cephalin"-calcium mixture*, freshly prepared before each test, was composed of equal parts of 0.05 M calcium chloride and a crude chloroform extract of acetone-dried rabbit brain (26), diluted from its stock solution 4,000- to 8,000-fold with buffer. The stock solution was stored at −25° C.

The effect of incubating crude Hageman factor with plasma thromboplastin antecedent was tested by incubating a mixture of 0.1 ml of each, suitably diluted with buffer, in silicone-coated tubes for the time desired. The tubes were then chilled in an ice bath. Simultaneously, each fraction was incubated separately for the same interval, chilled, and mixed in the same proportions. One-tenth ml of PTA-deficient plasma and 0.1 ml of the "cephalin"-calcium mixture were added to each tube and the "cephalin"-fortified clotting time was measured in the manner described. Conditions were so arranged that all tubes were assayed for clot-promoting activity simultaneously.

Inhibition of the activation of plasma thromboplastin antecedent was studied by adapting the preceding technique. One-tenth ml of crude Hageman factor, diluted 1 : 10, was incubated in a silicone-coated tube for 10 minutes at 37° C with 0.2 ml of the substance to be tested. Then 0.1 ml of undiluted crude PTA was added and the mixture was incubated for 30 additional minutes, chilled, and diluted with 9.6 ml of buffer. At the same time, 0.1 ml of Hageman factor was incubated for 10 minutes with 0.2 ml of buffer and for 30 additional minutes with 0.1 ml of PTA. Then 0.2 ml of the test substance and 9.4 ml of buffer were added to the mixture. The clot-promoting properties of the two mixtures were compared by measuring the effect of 0.1 ml aliquots upon the "cephalin"-fortified clotting time of normal plasma. As controls, the test substances, the clotting factors, and a mixture of Hageman factor and PTA were assayed alone. The substances tested included 2 M *sodium chloride; soybean trypsin inhibitor* (Nutritional Biochemicals) dissolved in buffer in a concentration of 1 mg per ml; ε-amino-*n-caproic acid* (Mann), dissolved in water in a concentration of 0.3 M; and 0.02 M *trisodium ethylenediamine tetraacetic acid*[6] prepared by neutralizing a 0.1 M solution of the disodium compound with sodium hydroxide to pH 7.3 and diluting it to 0.02 M with 1 part of water and 3 parts of buffer.

The *esterase* activity of crude Hageman factor and of PTA activated by Hageman factor was tested upon a substrate of 0.02 M *p*-toluenesulfonyl-L-arginine methyl ester for 24 hours at 37° C, using a previously published method (27). The concentration of Hageman factor in the enzyme-substrate mixture was one-tenth that of the plasma from which it was separated. The activated PTA had been prepared by incubating an equal volume of undiluted PTA and Hageman factor, diluted 1 :100,

[4] Russell's viper venom for this study was furnished through the kindness of Burroughs, Wellcome and Co., Tuckahoe, N. Y.

[5] Bovine fibrinogen was provided through the courtesy of Warner-Chilcott Labs., Morris Plains, N. J.

[6] Sequestrene Na_2 supplied through the courtesy of Alrose Chemical Co.

for 3 hours at 37° C. This mixture was diluted 10-fold in the enzyme-substrate mixture.

Centrifugations were at 2° C unless otherwise noted. Dialyses were carried out at 4° C in Visking No-Jax casings. Pipettes were washed in chromic acid; all other glassware was cleaned with Dreft. All assays were performed in duplicate in uncoated or silicone-coated Pyrex tubes (ID 8 mm); the average clotting time is recorded in the tables or figures. The protein content of plasma or fractions of plasma was measured by the technique of Lowry, Rosebrough, Farr and Randall (28). Unless otherwise specified, the buffer used in these studies was composed of 0.025 M barbital and 0.125 M sodium chloride at pH 7.5 (1).

RESULTS

I. The preparation of Hageman factor from PTA-deficient plasma. The distinction between PTA and the Hageman factor was originally made by observing that PTA-deficient plasma corrected the defect in plasma thought to be deficient in Hageman factor (1). Previously described preparations of Hageman factor, derived from pooled normal serum, were contaminated with PTA. The use of PTA-deficient plasma as a source for Hageman factor was therefore explored. PTA-deficient plasma appeared to contain normal amounts of Hageman factor, since it was as effective as normal plasma in shortening the recalcified clotting time of plasma deficient in this substance. The same PTA-deficient plasma retained its Hageman factor activity after it had been adsorbed with dilute aluminum hydroxide gel. A variable portion of Hageman factor activity, often as much as three-fourths, was removed by adsorption with concentrated aluminum hydroxide gel. Hageman factor could also be adsorbed virtually quantitatively onto carboxymethylcellulose, from which it could readily be eluted.

The corrective effect of PTA-deficient plasma for Hageman trait resisted heating at 56° C for 30 minutes. Heating PTA-deficient plasma at 60° C for 30 minutes destroyed virtually all of its Hageman factor activity.

PTA-deficient plasma, adsorbed with aluminum hydroxide gel, and heated at 56° C for 30 minutes, was fractionated with ammonium sulfate. Almost all Hageman factor activity was concentrated in the fraction soluble at 30 per cent saturation and insoluble at 50 per cent saturation with ammonium sulfate; a sharper separation could not be made by this technique. Small amounts of Hageman factor-like activity were also present in the fraction insoluble at 30 per cent saturation, and in the fraction soluble at 50 per cent but insoluble at 60 per cent saturation.

Serum prepared from PTA-deficient blood was as effective as normal serum in shortening the recalcified clotting time of plasma deficient in Hageman factor. In contrast, serum prepared from blood deficient in Hageman factor had only traces of clot-promoting activity for plasma deficient in the same factor. In these experiments, the serum was first adsorbed with barium sulfate to diminish the concentration of other clot-promoting substances.

The various properties that have been described were given consideration in the preparation of crude Hageman factor from PTA-deficient plasma.

II. Properties of crude Hageman factor. Crude Hageman factor, prepared from PTA-deficient plasma in the manner described, corrected the abnormality in plasma deficient in this substance, On the other hand, it was virtually without effect on the recalcified clotting time or "cephalin"-fortified clotting time of PTA-deficient plasma (Table I).

Previous studies have localized the defect in Hageman trait to the earliest stages of clotting. The properties of crude Hageman factor are in agreement with this interpretation. It accelerated the coagulation of normal plasma in glass tubes, but its effect was more dramatic in silicone-coated tubes (Table II). Its clot-promoting effect was a double logarithmic function: a linear relationship could be plotted between the logarithm of its con-

TABLE I

The effect of crude Hageman factor upon the coagulation of abnormal plasmas

Concentration of Hageman factor	Clotting time*	
	Hageman factor-deficient plasma	PTA-deficient plasma
	min	
1:10 plasma concentration	9	>60
1:40 plasma concentration	10	>60
Buffer control	>60	>60

* Clotting time of a mixture of 0.1 ml each of crude Hageman factor deficient in PTA, platelet-deficient plasma and "cephalin"-calcium mixture. The crude Hageman factor was diluted with buffer. Clotting times were measured in duplicate at 37° C in silicone-coated tubes.

TABLE II

The effect of activated Hageman factor and of spontaneously active plasma thromboplastin antecedent upon the coagulation of normal plasma

Preliminary period of incubation	Clotting time* (min) of normal plasma upon the addition of:		
	A) Hageman factor	B) PTA	C) Buffer
0	13	13	
1	11	14	
2	10	14	
4	10	14	
8	9	16	
16	11	15	
32	13	15	
			23

* Clotting time of a mixture of 0.1 ml each of crude activated Hageman factor, deficient in PTA (at 1:100 plasma concentration) or spontaneously activated crude PTA, deficient in Hageman factor (at original plasma concentration) or buffer, normal plasma and "cephalin"-calcium mixture. The Hageman factor or PTA was incubated alone at 37° C in silicone-coated tubes. At intervals, the plasma was added to successive tubes. After 32 minutes, the "cephalin"-calcium mixture was added to all tubes and the clotting times were measured in duplicate at 37° C.

centration and the logarithm of the clotting time of the substrate plasma.

The clot-promoting property of crude Hageman factor suggested that it had been activated at least in part during its preparation. The clot-promoting effect of this "activated" Hageman factor was increased by incubating it with normal plasma before recalcification. In one such experiment, small amounts of crude activated Hageman factor were mixed with normal plasma and incubated in silicone-coated tubes at 37° C. At intervals, aliquots of this mixture were recalcified and the clotting time was measured in the presence of traces of "cephalin." Under these conditions, the clotting time of the mixture of activated Hageman factor and normal plasma gradually shortened (Table II, columns A and C). These experiments demonstrated that crude activated Hageman factor reacted with a component of normal plasma before the addition of calcium. After 8 minutes, the clotting time of the mixture gradually lengthened. The significance of this effect will be discussed later.

The clot-promoting effect of activated Hageman factor was apparently exerted at an early step in blood coagulation. Preparations of activated Hageman factor were devoid of detectable thrombin-like activity, since they did not clot solutions of fibrinogen even in the presence of calcium ions. They were without effect upon the one-stage prothrombin time of normal plasma. Nonetheless, they greatly increased the rate at which prothrombin was converted to thrombin during the coagulation of normal blood (Table III).

The increased prothrombin "consumption" which occurred in the presence of crude activated Hageman factor could not be attributed to its contamination with other recognized clotting factors. Preparations of Hageman factor were free of detectable pro-SPCA, Stuart factor, proaccelerin, prothrombin and antihemophilic factor, and did not shorten the abnormally long prothrombin time of the plasma of a patient under treatment with Dicumarol. One undiluted preparation shortened the recalcified clotting time of plasma deficient in Christmas factor from 70 to 41 minutes; in contrast, normal plasma, diluted 1:40, shortened the clotting time of the same plasma to 7 minutes. Hageman factor was thus not contaminated with any appreciable amount of other known components of the clotting system.

At least three hydrolytic enzymes found in human plasma—plasmin, thrombin and the esterase derived from the first component of complement—digest *p*-toluenesulfonyl-L-arginine methyl ester. Crude activated Hageman factor did not hydrolyze this substrate, distinguishing it from these enzymes. However, the preparation did contain plasminogen, since fibrinolytic activity appeared upon the addition of streptokinase.

III. The effect of carboxymethylcellulose upon blood coagulation. Contact with glass or certain other adsorbent surfaces accelerates the clotting of normal plasma. This clot-promoting activity can be detected only in plasmas containing Hage-

TABLE III

The effect of activated Hageman factor upon serum prothrombic activity

Test substance	Prothrombic activity*
	%
Activated Hageman factor	5
Buffer	35

* Serum prothrombic activity of a mixture of 0.1 ml of crude activated Hageman factor (deficient in PTA), diluted 100-fold with buffer, or buffer, and 2.15 ml of whole blood, incubated at 37° C for 30 minutes in Pyrex tubes.

man factor. Contact with these adsorbents seems to transform Hageman factor from an inactive to an active form in which it can accelerate clotting. Evidence has been presented that the crude Hageman factor used in the present study had been activated at least in part (Section II and Table II). Since its adsorption to carboxymethylcellulose was a step in the preparation of crude activated Hageman factor, the possibility was considered that carboxymethylcellulose, like other adsorbents, accelerates the clotting of normal plasma. To test this, normal plasma was agitated with carboxymethylcellulose and then tested for its clot-promoting effect on untreated plasma (Table IV). This experiment demonstrated that agitation with carboxymethycellulose induced clot-promoting activity in normal plasma.

IV. The preparation of PTA from Hageman factor-deficient plasma. No satisfactory method of preparing PTA from normal plasma has been described. The plasma of patients with Hageman trait contains PTA activity. The properties of such plasma were studied to determine the conditions needed to prepare PTA that was deficient in other recognized clotting factors.

Plasma deficient in Hageman factor shortened the recalcified clotting time of PTA-deficient plasma to the same degree as did normal plasma, as if it contained normal amounts of PTA. Similarly, serum obtained from patients with Hageman trait shortened the clotting time of PTA-deficient plasma, but serum prepared from PTA-deficient blood was without any corrective effect. Each serum had first been adsorbed with barium sulfate to remove other clot-promoting substances. PTA activity was also present in oxalated Hageman trait plasma which had been stored at − 25° C for more than 4 years.

TABLE IV

The clot-promoting effect of carboyxmethylcellulose

Test plasma	Clotting time*
	min
Carboxymethylcellulose-treated plasma	24
Control plasma	>300

* Clotting time of a mixture of 0.1 ml each of the test plasma, untreated plasma and 0.05 M calcium chloride. The test plasma was prepared by agitating 1 ml of normal plasma for 10 minutes at room temperature in a Lusteroid tube containing 75 mg of carboxymethylcellulose, which was then removed by centrifugation; the control plasma was similarly treated except that the carboxymethylcellulose was omitted. Clotting times were measured in duplicate at 37° C in silicone-coated tubes.

Adsorption of normal plasma or plasma deficient in Hageman factor with dilute aluminum hydroxide gel usually resulted in a decrease in its PTA activity of 75 to 80 per cent. When the adsorption was carried out with concentrated aluminum hydroxide gel, nearly all of the corrective effect of these plasmas was lost. A second adsorption with concentrated aluminum hydroxide usually removed all but traces of PTA-like activity. Thus, this factor was more readily adsorbed from plasma by aluminum hydroxide than was Hageman factor. Attempts to elute PTA from aluminum hydroxide gel have been unsuccessful.

PTA could also be adsorbed onto carboxymethylcellulose; in this case, it could be eluted without obvious loss.

Normal plasma or plasma deficient in Hageman factor, heated at 56° C for 30 minutes, had only about one-fourth or less of its original ability to shorten the clotting time of PTA-deficient plasma. All activity was lost after incubation at 60° C for 30 minutes, and plasma, heated in this way, actually acquired clot-inhibitory properties. When plasma was successively adsorbed with dilute aluminum hydroxide gel and heated at 56° C for 30 minutes, the loss of PTA-like activity was no greater than when either procedure was performed separately. Normal plasma, treated in this way, still shortened the clotting time of plasma deficient in Hageman factor almost as readily as did untreated plasma. On the other hand, Hageman factor-deficient plasma, treated the same way, had no effect upon untreated Hageman factor-deficient plasma. Such plasma was suitable as a starting material for the concentration of PTA, deficient in Hageman factor.

Maximal PTA activity was found in the fraction of adsorbed, heated plasma soluble at 40 per cent and insoluble at 50 per cent saturation. However, the degree of purification achieved by ammonium sulfate precipitation at this stage was not enough to be useful in purification.

Crude PTA, deficient in other clotting factors and apparently suitable for the experiments to be described, was prepared by methods derived from these observations.

V. Properties of crude plasma thromboplastin antecedent. Freshly prepared crude PTA, de-

TABLE V

The effect of crude plasma thromboplastin antecedent upon the coagulation of PTA-deficient plasma

Test mixture	Clotting time*
	min
Hageman factor-deficient plasma, diluted 1:20	10
Hageman factor-deficient plasma, diluted 1:80	13
Hageman factor-deficient plasma, diluted 1:320	15
Crude PTA, 1:20 plasma concentration	14
Buffer control	18

* Clotting time of a mixture of 0.1 ml each of Hageman factor-deficient plasma or crude PTA (prepared from Hageman factor-deficient plasma) or buffer, PTA-deficient plasma, and 0.025 M calcium chloride. The Hageman factor-deficient plasma and PTA were diluted with buffer. The clotting times were measured in duplicate at 25° C in Pyrex tubes.

rived from Hageman factor-deficient plasma, shortened the clotting time of PTA-deficient plasma in Pyrex tubes (Table V). On the other hand, the same fraction was either without effect on normal plasma or was slightly inhibitory (Table VI). The significance of this inhibitory activity is not yet clear. An inhibitor of the clot-promoting properties of glass and other adsorbents has been found in Hageman factor-deficient plasma (1, 4, 10, 29), but its identity with the inhibitor in crude PTA is uncertain.

Crude PTA did not seem to be contaminated with other recognized clotting factors. It lacked thrombin-like activity and did not influence the one-stage prothrombin time of normal blood. It did not influence the conversion of prothrombin to thrombin in whole blood, as tested by mixing 0.2 ml of an undiluted preparation to 2.15 ml of whole blood and measuring the prothrombin remaining after 20 minutes. It contained no measurable pro-SPCA, Stuart factor, proaccelerin, prothrombin, antihemophilic factor or Christmas factor, and was without influence on the prothrombin time of the plasma of a patient under treatment with Dicumarol.

TABLE VI

The effect of crude plasma thromboplastin antecedent upon the coagulation of normal plasma

Test mixture	Clotting time*
	min
Crude PTA	23
Buffer control	16

* Clotting time of a mixture of 0.1 ml each of crude PTA or buffer, normal plasma and "cephalin"-calcium mixture. The PTA was prepared from Hageman factor-deficient plasma and was at its original concentration in plasma. Clotting times were measured in duplicate at 37° C in silicone-coated tubes.

VI. The effect of crude activated Hageman factor upon plasma thromboplastin antecedent. When crude preparations of activated Hageman factor and of PTA were incubated together, clot-promoting activity developed. The experiments to be described suggest that in this reaction the clot-promoting activity evolved from the action of activated Hageman factor upon PTA.

Crude preparations of activated Hageman factor and of PTA were incubated in silicone-coated tubes at 37° C. At intervals, aliquots were added to a substrate of plasma deficient in PTA, and the clotting time of the mixture was measured in silicone-coated tubes. This experimental design was chosen to allow the study of any interreaction of activated Hageman factor and PTA without confusion by their effect upon the substrate plasma. Thus, PTA was furnished in the incubation mixture but not in the substrate. Hageman factor was present both in the incubation mixture and in the substrate. However, the crude Hageman factor in the incubation mixture had been "ac-

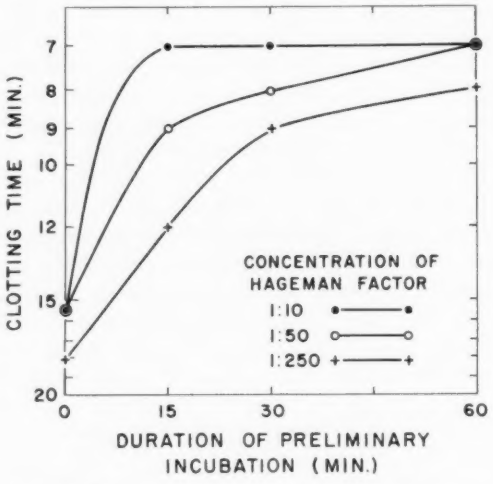

FIG. 2. THE EVOLUTION OF CLOT-PROMOTING ACTIVITY IN MIXTURES OF CRUDE ACTIVATED HAGEMAN FACTOR AND PTA. THE INFLUENCE OF CONCENTRATION OF HAGEMAN FACTOR. Clotting time of a mixture of 0.1 ml each of crude activated Hageman factor diluted serially with buffer, crude PTA diluted 1:4, PTA-deficient plasma and "cephalin"-calcium mixture. The activated Hageman factor and PTA were incubated for the indicated intervals prior to the addition of plasma and "cephalin"-calcium mixture. Clotting times were determined in duplicate in silicone-coated tubes at 37° C.

tivated" during its preparation while that in the substrate plasma was presumably largely inactive, since this had not had contact with glass. Qualitatively similar results were obtained in experiments in which normal plasma was used as a substrate, but quantitative measurement was much more difficult.

When activated Hageman factor was incubated with PTA in silicone-coated tubes, potent clot-promoting activity evolved. The *rate* at which clot-promoting activity developed was determined by the concentration of crude Hageman factor in the mixture (Figure 2). At the highest concentration of activated Hageman factor tested, clot-promoting activity was maximal within 15 minutes after this substance was mixed wtih PTA. With lesser concentrations of activated Hageman factor, clot-promoting activity developed at a slower rate but, ultimately, essentially the same clot-promoting activity evolved regardless of the concentration of Hageman factor tested.

On the other hand, the *magnitude* of the clot-promoting activity which evolved was a function of the concentration of PTA in the mixture (Figure 3). In this experiment, an amount of activated Hageman factor sufficient to give a maximal effect within 15 minutes was incubated for 60 minutes with different amounts of PTA. In this way, conditions were arranged so that clot-promoting activity was assayed only after it had reached full development. Under these circumstances, clot-promoting activity developed in relation to the concentration of PTA.

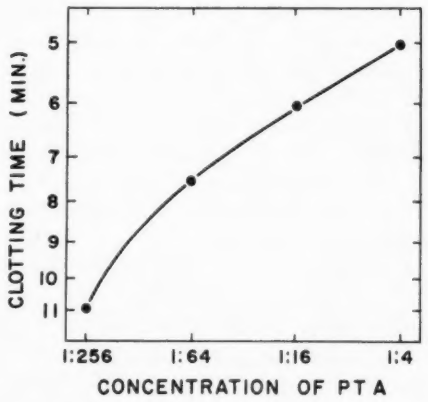

FIG. 3. THE EVOLUTION OF CLOT-PROMOTING ACTIVITY IN MIXTURES OF CRUDE ACTIVATED HAGEMAN FACTOR AND PTA. THE INFLUENCE OF CONCENTRATION OF PTA. Clotting time of a mixture of 0.1 ml each of crude activated Hageman factor diluted 1:10, crude PTA diluted serially with buffer, PTA-deficient plasma and "cephalin"-calcium mixture. The activated Hageman factor and PTA were incubated at 37° C for 60 minutes prior to the addition of plasma and "cephalin"-calcium mixture. Clotting times were determined in duplicate at 37° C.

TABLE VII

The spontaneous appearance of clot-promoting activity in preparations of plasma thromboplastin antecedent

Test mixture	Clotting time* (min) At 0 hrs	After 24 hrs	After 48 hrs
PTA incubated at 4° C	23	15	22
PTA incubated at 25° C		11	13
PTA incubated at 37° C		8	9
Buffer control	16	17	18

* Clotting time of a mixture of 0.1 ml each of crude PTA or buffer, normal plasma and "cephalin"-calcium mixture. Five-tenths ml portions of freshly prepared PTA (prepared from Hageman factor-deficient plasma) were incubated in silicone-coated tubes at 4°, 25° or 37° C and aliquots were removed for testing at 0, 24, and 48 hours. Clotting times were measured in duplicate at 37° C in silicone-coated tubes.

The evolution of clot-promoting activity in a mixture of crude activated Hageman factor and crude PTA was not inhibited by the presence of 0.01 M trisodium ethylenediamine tetraacetic acid, 0.15 M ε-amino-*n*-caproic acid, 0.01 M benzoyl-L-arginine methyl ester, or soybean trypsin inhibitor in a concentration of 0.5 mg per ml. However, clot-promoting activity did not develop in the presence of 1 M NaCl.

VII. The appearance of clot-promoting activity in crude preparations of plasma thromboplastin antecedent. That PTA may be converted into a clot-promoting substance was unexpectedly corroborated in studies of crude PTA prepared from plasma deficient in Hageman factor. Under certain conditions, clot-promoting activity arose spontaneously in preparations of crude PTA; this activity could not be distinguished from that which appeared when crude Hageman factor and PTA were incubated together.

Crude PTA was incubated in silicone-coated tubes in the presence of toluene vapor. At the start, and 24 and 48 hours later, the fraction was tested for clot-promoting activity (Table VII). Prior to incubation the fraction delayed the "cephalin"-fortified clotting time of normal plasma.

After incubation at 37° C for 24 hours, the same fraction shortened the clotting time; no further activity developed in 48 hours. Another sample, incubated at 25° C, had a lesser clot-promoting effect, and a third sample, incubated at 4° C, was virtually unchanged.

The "spontaneous" appearance of clot-promoting activity in crude preparations of PTA was erratic, and the factors responsible for its development could not be clarified. In contrast to that which developed when PTA was incubated with crude activated Hageman factor, clot-promoting activity never appeared in less than 8 hours. Spontaneous clot-promoting activity developed in preparations of PTA derived from each of the five patients studied who had Hageman trait. Activity was more likely to appear when the fraction was dissolved in a volume of buffer equal to that of the plasma from which it was prepared; little or no activity evolved when the fraction was diluted further. Although activity evolved more consistently at 37° C, storage of the fraction at temperatures as low as −25° C did not always prevent its appearance within a few weeks. In several experiments, attempts were made to concentrate crude PTA by precipitation with ammonium sulfate at 55 per cent saturation followed by dialysis against buffer. Nearly always, clot-promoting activity appeared in these fractions, although the stage of preparation at which it developed could not be distinguished. Clot-promoting activity also appeared when crude PTA was adsorbed onto calcium phosphate gel and then eluted.

VIII. Evidence that the spontaneous clot-promoting activity of PTA preparations was not due to the action of activated Hageman Factor. The clot-promoting activity that appeared "spontaneously" in preparations of crude PTA accelerated the clotting not only of normal plasma but also of plasma obtained from patients with Hageman trait or PTA-deficiency (Table VIII). However, several lines of evidence indicate that this clot-promoting activity was probably not due to contamination of the crude PTA with Hageman factor. PTA was adsorbed more readily than Hageman factor by concentrated aluminum hydroxide gel (Section IV); a sample of Hageman factor-deficient plasma, adsorbed with diluted aluminum hydroxide gel, had PTA activity, but the same plasma, adsorbed with concentrated aluminum hydroxide gel, lacked PTA activity.

TABLE VIII

The clot-promoting effect of spontaneously activated plasma thromboplastin antecedent

Test mixture	Clotting time* (min)		
	Normal plasma	Hageman factor-deficient plasma	PTA-deficient plasma
Spontaneously activated PTA	8	7	8
Buffer control	31	>60	>60

* Clotting time of a mixture of 0.1 ml spontaneously activated crude PTA (prepared from Hageman factor-deficient plasma) or buffer, plasma and "cephalin"-calcium mixture. Clotting times were measured in duplicate at 37° C in silicone-coated tubes.

The removal of PTA from plasma deficient in Hageman factor simultaneously removed its capacity to generate clot-promoting activity. In one experiment, Hageman factor-deficient plasma was adsorbed twice with concentrated aluminum hydroxide gel; it no longer contained detectable PTA, as measured by its capacity to shorten the clotting time of PTA-deficient plasma. This adsorbed plasma was heated at 56° C for 30 minutes and adsorbed with carboxymethylcellulose in the usual way. The eluate of the carboxymethylcellulose was incubated in silicone-coated tubes at 37° C for 91 hours. At the end of this time, it contained only about 2 per cent of the clot-promoting activity of an eluate prepared from the same plasma that had been adsorbed with dilute rather than concentrated aluminum hydroxide gel.

The clot-promoting activity which appeared in crude PTA was differentiated from Hageman factor by a second method. Spontaneously active PTA was qualitatively different from crude activated Hageman factor. It will be recalled that the clotting time of normal plasma was shortened by preliminary incubation with activated Hageman factor (Table II, column A). Under the same conditions, preliminary incubation with normal plasma did not increase the clot-promoting properties of spontaneously active PTA (column B).

In still another experiment, a preparation of crude activated Hageman factor was diluted until its clot-promoting activity for normal plasma was equal to that of a "spontaneously" active preparation of crude PTA. A mixture of activated Hageman factor and freshly prepared, inactive PTA was incubated at 37° C for 80 minutes. At the

TABLE IX

The effect of activated Hageman factor and of spontaneously activated plasma thromboplastin antecedent upon inactive PTA

Test mixture	Clotting time*
	min
Inactive PTA, 0.1 ml + buffer, 0.1 ml	19
Active PTA, 0.1 ml + buffer, 0.1 ml	12
Activated Hageman factor, 0.1 ml + buffer, 0.1 ml	11
Inactive PTA, 0.1 ml + active PTA, 0.1 ml	12
Inactive PTA, 0.1 ml + activated Hageman factor, 0.1 ml	7
Buffer control, 0.2 ml	17

* Clotting time of 0.2 ml test mixture, 0.1 ml normal plasma and 0.1 ml "cephalin"-calcium mixture. The test mixtures were incubated in silicone-coated tubes for 80 minutes at 37° C prior to the addition of plasma and "cephalin"-calcium. Clotting times were measured in duplicate at 37° C.

end of this period, further clot-promoting activity had evolved in the mixture (Table IX). On the other hand, no additional clot-promoting activity appeared in a mixture of "spontaneously active" and inactive PTA incubated for the same period. Again, spontaneously active PTA did not behave like activated Hageman factor in its effect upon inactive PTA.

IX. Some properties of the clot-promoting activity evolving in crude plasma thromboplastin antecedent "spontaneously" or upon treatment with activated Hageman factor. The clot-promoting activity which appeared when crude PTA was treated with activated Hageman factor could not be distinguished from that which evolved "spontaneously." In preparations that seemed to be fully activated, the clot-promoting activity was striking. Either preparation, diluted 300-fold or more compared with the plasma from which it was prepared, significantly accelerated the "cephalin"-fortified clotting time of an equal volume of normal plasma.

The clot-promoting activity could not be identified with that of other known clotting factors. Neither preparation was thrombin-like, for they did not clot bovine fibrinogen, even in the presence of calcium ions. Nor did they contain prothrombin, proaccelerin, pro-SPCA, Stuart factor or antihemophilic factor, as tested by specific assays for each of these substances. They either were without corrective effect upon plasma deficient in Christmas factor, or shortened its recalcified clotting time or partial thromboplastin time insignificantly; in one experiment, the partial thromboplastin time of plasma deficient in Christmas factor was shortened from 653 to 553 seconds by a preparation of PTA activated by Hageman factor. The partial thromboplastin time of normal plasma, tested by this method, is usually less than 100 seconds.

By inference, the clot-promoting property appeared to function early in the clotting process. It altered neither the thrombin time nor the prothrombin time of normal plasma. However, it significantly increased prothrombin "consumption" in whole blood. Typical data, obtained with a "spontaneously" active PTA preparation, are recorded in Table X. These experiments are compatible with the view that the clot-promoting substance evolved from preparations of PTA acts early in the clotting process.

The clot-promoting agent which developed when PTA was treated with Hageman factor did not digest *p*-toluenesulfonyl-L-arginine-methyl ester, distinguishing its action from that of thrombin, plasmin and the esterase derived from the first component of complement.

X. The inhibition of activated Hageman factor and "activated" plasma thromboplastin antecedent by plasma. The clot-promoting activity which appears when normal plasma is exposed to glass gradually deteriorates (30). This decrease in activity has been attributed to the presence of an inhibitory substance that may have enzymatic properties (2). The specificity of the inhibition with regard to activated Hageman factor or activated plasma thromboplastin is not certain.

TABLE X

The effect of spontaneously activated plasma thromboplastin antecedent upon serum prothrombic activity

Test substance	Prothrombic activity*
	%
Inactive PTA	50
Spontaneously active PTA	10
Buffer control	50

* Serum prothrombic activity of a mixture of 0.2 ml of undiluted inactive or spontaneously active PTA or buffer and 2.05 ml whole blood, incubated at 37° C for 1 hour in Pyrex tubes. The PTA was prepared from Hageman factor-deficient plasma.

A fraction of plasma was prepared which inhibited the clot-promoting activity of glass-treated plasma. This fraction was incubated with crude activated Hageman factor and with "spontaneously" activated PTA. In each case, the clot-promoting activity was decreased (Table XI). Whether the same principle inactivated Hageman factor and "spontaneously" activated PTA was not ascertained. Additional studies to confirm the enzymatic nature of the inhibitory substance were not performed, but its activity was not diminished by incubation with diisopropyl fluorophosphate at a concentration of 5×10^{-4} M, 0.05 mg soybean trypsin inhibitor per ml, 0.001 N mercuric chloride, 0.0125 N hydrogen peroxide, or 0.01 M trisodium ethylenediamine tetraacetic acid.

XI. The effect of diisopropyl fluorophosphate upon Hageman factor, plasma thromboplastin antecedent and activated plasma thromboplastin antecedent. Becker (31) observed that diisopropyl fluorophosphate inhibited the clot-promoting properties of Hageman factor. However, the preparation which he used undoubtedly contained PTA in addition to Hageman factor, and his method of assay did not distinguish between these factors. Crude activated Hageman factor, devoid of PTA, was incubated with DFP at a concentration of 5×10^{-4} M for 1 hour and then dialyzed against repeated changes of buffer. Similarly, crude PTA, devoid of Hageman factor, and PTA, activated "spontaneously" or by incubation with activated Hageman factor, were treated with DFP.

TABLE XI

The effect of an inhibitory fraction of plasma upon activated Hageman factor and spontaneously activated plasma thromboplastin antecedent

Test mixture	Clotting time*
	min
Activated Hageman factor, 0.1 ml+inhibitor, 0.1 ml, separately incubated, then mixed	9
Activated Hageman factor, 0.1 ml+inhibitor, 0.1 ml, incubated together	13
Activated PTA, 0.1 ml+inhibitor, 0.1 ml, separately incubated, then mixed	11
Activated PTA, 0.1 ml+inhibitor, 0.1 ml, incubated together	19
Buffer, 0.1 ml+inhibitor, 0.1 ml	18
Buffer, 0.2 ml	17

* Clotting time of a mixture of 0.2 ml of the test mixture, 0.1 ml of normal plasma and 0.1 ml of "cephalin"-calcium mixture. The test mixture was composed of crude activated Hageman factor (diluted 1:10) or crude spontaneously activated PTA, mixed with an inhibitory fraction of plasma. These substances were incubated either together or separately and then combined. This incubation and the clotting times were performed in duplicate at 37° C in silicone-coated tubes.

TABLE XII

The effect of diisopropyl fluorophosphate upon activated Hageman factor and activated plasma thromboplastin antecedent

Test mixture	Clotting time*	
	Hageman factor-deficient plasma	PTA-deficient plasma
	min	
Untreated activated PTA		11
DFP-treated activated PTA		16
Untreated activated Hageman factor	14	
DFP-treated activated Hageman factor	22	
Buffer control	65	15

* Clotting time of a mixture of 0.1 ml each of crude activated Hageman factor or PTA (each diluted 1:10), plasma and 0.025 M calcium chloride. The clotting times were measured in duplicate in Pyrex tubes at 25° C.

Treated and untreated fractions were then tested for their specific corrective effect for deficient plasmas, and for their nonspecific clot-promoting activity for normal plasma.

Crude activated Hageman factor, devoid of PTA, regularly lost three-fourths or more of its clot-promoting activity for both normal plasma and plasma deficient in Hageman factor after treatment with DFP (Table X). In contrast, when normal plasma was incubated for 1 hour with DFP and then dialyzed against buffer, none of its Hageman factor-like activity was lost, and clot-promoting activity appeared when it was agitated with crushed glass. In normal plasma, Hageman factor behaves as if it were inactive, until it is exposed to glass or similar substances. One may infer that DFP inhibits activated Hageman factor but not its inactive precursor.

Similarly, crude PTA was unaffected by incubation with DFP. However, "spontaneously" active PTA, or PTA activated by Hageman factor, was sensitive to DFP, losing about three-fourths of its activity under the conditions at which it was tested (Table XII).

DISCUSSION

Almost 100 years ago, Lister (32) observed that the coagulation of mammalian blood was enhanced by contact with a foreign surface. Normal blood—liquid within the blood vessels—clots within a few minutes in a glass tube. The mechanisms through which glass and other clot-promoting surfaces act have been studied persistently. Until recently, attention was centered upon the effect of these agents on the formed elements of

the blood. However, a profusion of experiments, beginning with those of Bordet and Gengou (33), demonstrated that glass and certain other substances can initiate or accelerate clotting in plasma depleted of its cells. One site of action of glass is Hageman factor, present in normal plasma but lacking in the plasma of patients with Hageman trait (2–8). Perhaps other factors are altered as well (7, 34); it is difficult to distinguish between a direct effect of glass upon a clotting factor and an indirect effect mediated through its activation of Hageman factor.

Currently, the most satisfactory hypothesis to explain the effect of glass upon Hageman factor relates to its adsorbent properties. Clotting is accelerated by many other adsorbents including kaolin (2), barium carbonate (2, 35), charcoal (2), Supercel (2), Celite (5), bentonite (36), asbestos (8) and silicic acid (37), and, as demonstrated in the present study, carboxymethylcellulose. Like glass, these adsorbents are effective only when the plasma tested contains Hageman factor. These considerations have led to the view that the clot-promoting properties of glass and other adsorbents depend upon their capacity to adsorb Hageman factor, which changes from an inactive to an active form in the process.

The molecular rearrangements that result in the activation of Hageman factor are unknown. Fiala (35) thought that glass or barium carbonate initiated clotting by removing an inhibitor from plasma. We have been unable to isolate an inhibitor from untreated normal plasma. However, plasma deficient in Hageman factor inhibits the clot-promoting effects of glass, barium carbonate and asbestos (2, 29). This inhibitory activity appears to be directed against the *activation* of Hageman factor. Minute amounts of preparations containing *active* Hageman factor shorten the clotting time of plasma deficient in this substance (1) (Table I). Attractive as it may be to imagine that the same inhibitory activity exists in normal plasma, masked by the presence of Hageman factor, this view is unproved. Similar inhibitory activity can be demonstrated in normal plasma heated at 60° C to destroy the Hageman factor it contains, but these results may well be artifactual (38). Dissenting views, that the inhibitory activity in Hageman factor-deficient plasma is "nonspecific" (10) or is the essential defect in Hageman trait (11), are equally conjectural.

Once it has been activated, Hageman factor apparently accelerates the coagulation of normal blood or plasma (Table II). Previous studies to determine how this acceleration comes about were handicapped because preparations of Hageman factor of adequate purity were not available. Our earlier attempts to purify Hageman factor from normal serum (2, 39) were vitiated by unsuspected contamination with plasma thromboplastin antecedent (12). Nonetheless, several groups of investigators have proposed that Hageman factor, once activated by glass, reacts with a second clotting factor, PTA, to produce a clot-promoting substance variously called activation product (5), the third prothromboplastic factor (11), antihemophilic factor C (11) or activated PTA (10). Hardisty and Margolis (10) conceived that active Hageman factor serves as a catalyst to convert PTA from an inactive to an active form, but they did not provide direct supporting evidence. They believed that Hageman factor exerts its effect upon PTA only while both are adsorbed to glass or other surfaces. In their view, the activated PTA then returns to solution to initiate further stages in the clotting process. All agree that calcium ions are not required for the activation of Hageman factor by glass, but Soulier and Prou-Wartelle (36) thought that calcium might be needed for the interaction of Hageman factor and PTA.

Some clarification of the function of activated Hageman factor and its relationship to PTA has been possible, using the relatively simple techniques described in the present report. A crude fraction, rich in activated Hageman factor and apparently deficient in other known clotting factors, was prepared from plasma believed deficient in PTA. In the same way, a crude fraction containing PTA was prepared from plasma deficient in Hageman factor. Hageman factor, prepared by this method, was apparently purified about 100-fold and in the process was activated, for now it accelerated the clotting of normal blood or plasma; whether all or only part of the Hageman factor was activated is not yet clear. The fraction corrected the coagulative defect of plasma deficient in Hageman factor but had no effect upon the clotting time of plasma deficient in PTA. Its effect on normal blood seemed to be localized to an early stage of

clotting, in keeping with earlier premises that the defect in Hageman factor-deficient plasma is in the initiation of blood coagulation and that Hageman factor is needed for the evolution of thromboplastic activity in shed blood (1).

Experiments in which crude activated Hageman factor was incubated with normal citrated plasma in silicone-coated tubes demonstrated that this factor reacted with some component of the plasma in the absence of calcium ions. However, they provided no clues concerning the component with which activated Hageman factor reacted.

When crude activated Hageman factor was incubated with crude PTA, clot-promoting activity of considerable potency gradually evolved. Kinetic experiments demonstrated that the extent of this activity was related to the concentration of PTA in the mixture, while the rate at which it developed depended upon the concentration of activated Hageman factor. Our present interpretation of these studies is that Hageman factor is an enzyme whose substrate, PTA, is converted to an active clot-promoting agent, tentatively designated as activated PTA. This reaction occurred when solutions of activated Hageman factor and PTA were incubated in silicone-coated tubes; the presence of a glass-like surface did not seem necessary. The reaction took place in the presence of appreciable quantities of trisodium ethylenediamine tetraacetic acid and thus did not seem to be calcium-dependent. Thus, the initial phases of blood coagulation may be schematized as in Figure 4.

Nothing in these experiments rules out the possibility that other as yet unidentified agents, present in the crude preparations of Hageman factor and PTA, are needed for the development of clot-promoting activity. Indeed, these fractions may well contain other clot-accelerating substances, which may function in ways not detected by our present techniques. A wealth of previous experience cautions against the assumption that "purified" clotting factors are free of other clot-accelerating materials.

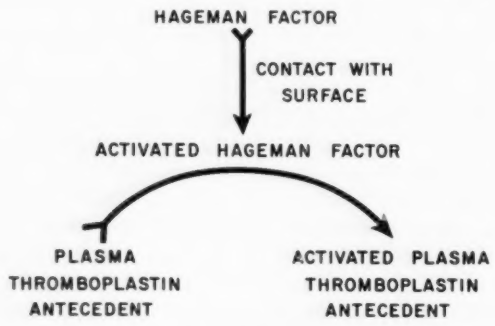

FIG. 4. A TENTATIVE SCHEME OUTLINING THE INITIAL STEPS IN THE COAGULATION OF PLASMA. Modified from Reference 47.

The enzymatic nature of activated Hageman factor is supported by its inhibition by diisopropyl fluorophosphate, first demonstrated by Becker (31) and now confirmed with purer preparations of activated Hageman factor. Likewise, the reaction of activated PTA with DFP supports the conclusion that it also is an enzyme. In contrast, Hageman factor and PTA are not affected by DFP. Thus, these reactions are analagous to those of trypsinogen and trypsin (40–42), chymotrypsinogen and chymotrypsin (40), plasminogen and plasmin (43), and prothrombin and thrombin (44, 45). In each of these cases, the enzyme precursors are unaffected by organophosphorous compounds, while the active form is rapidly inhibited. Whether activated Hageman factor and activated PTA preparations will show similar pH dependency as well as the direct binding of the diisopropyl phosphoryl moiety to a serine residue remains to be investigated. Neither activated Hageman factor nor activated PTA was totally inactivated by DFP. This probably reflects the presence in the crude preparations tested of other, unidentified clot-promoting substances which are DFP-resistant.

Activated Hageman factor may well have natural substrates other than PTA. Margolis (46, 47) has implicated this substance in the activation of "pain-producing factor" and "capillary permeability factor" of normal serum. However, a search for protein or synthetic substrates of activated Hageman factor, only partly recorded here, has been unrewarding.

Information has also been provided concerning the properties of PTA. The crude preparations we have made represent an apparent purification of this substance of only about 10- to 20-fold. In its inactive form it had no effect upon the coagulation of normal plasma or plasma deficient in Hageman factor. Once treated by activated Hageman factor, it accelerated the coagulation of both

of these plasmas. Again, its action seemed limited to the earliest steps of coagulation, the formation of thromboplastic activity. Presumably, activated Hageman factor affected this stage of coagulation by activating PTA, and this in turn initiated the steps leading to the conversion of prothrombin to thrombin. In these reactions, activated PTA may well have enzymatic activity; its inhibition by diisopropyl fluorophosphate is compatible with this view. However, its substrate has not been delineated. Preliminary experiments, not reported here, suggest that Christmas factor may be a substrate, but data to establish this are not yet available. Calcium ions may be needed for the action of activated PTA on its substrate, but again satisfactory experimental data are not yet at hand.

A perplexing question has been the dearth of hemorrhagic symptoms in persons whose plasma apparently lacks Hageman factor (1, 12). Some insight into this problem may have been gained from studies of "spontaneously" active PTA. Crude PTA, purified by the method described, did not accelerate the coagulation of normal blood or plasma. However, such preparations, incubated alone at 37° C, acquired clot-promoting activity that could not be distinguished from that which evolved in the presence of Hageman factor. By implication, some mechanisms permitting the activation of PTA at the site of an injury may be available to the patient with Hageman trait. How this might come about has not yet been studied.

The mechanism responsible for the spontaneous appearance of clot-promoting activity in crude preparations of PTA is unknown. Studies of this process have been hampered because as yet all of our attempts at further purification of PTA have produced activated products. As yet, there is no reason to believe that the activation process is autocatalytic and, indeed, the addition of activated PTA to its inactive precursor seemed without effect. Conceivably, the plasma from which the PTA was prepared, devoid of detectable Hageman factor by other measures, contained traces of this substance. Under appropriate conditions these traces of Hageman factor might activate PTA. Of course, other explanations must also be considered. Several groups have suggested that the plasma of patients with Hageman trait contains normal amounts of Hageman factor, blocked by the presence of an inhibitor. This assumption seems unwarranted, since the merest trace of normal plasma will shorten the clotting time of plasma from patients with Hageman trait.

The clot-promoting activity induced by treating normal plasma with glass is labile (30). The loss of clot-promoting activity is apparently related to the presence of one or more inhibitors. A crude concentrate of plasma, containing this inhibitory activity, seemed to inactivate both activated Hageman factor and spontaneously activated PTA. Whether this inhibitory activity is due to the presence of one or several substances is not yet clear. Further studies have been vitiated by the lability of the inhibitory activity during purification.

This discussion has purposely emphasized the large areas of uncertainty concerning the functions and properties of Hageman factor and PTA. It is hoped that these uncertainties will disappear when purer preparations of these factors become available. By taking advantage of the ease with which aluminum hydroxide adsorbs PTA we have made considerable progress in the purification of Hageman factor from normal plasma; our best preparations are now at least 1,000-fold purified, but the yields are poor. No such progress has been made in the purification of PTA; all attempts have been blocked by the spontaneous activation of this substance. As purer preparations become available, a more meaningful analysis of the successive steps in the coagulative process should become possible.

SUMMARY

Methods for the partial purification of Hageman factor and plasma thromboplastin antecedent, and some of the properties of these substances, are described. In the process of purification, Hageman factor seems to be converted from an inactive to an active form. When partially purified activated Hageman factor and plasma thromboplastin antecedent are incubated together, clot-promoting activity evolves. Studies suggest that plasma thromboplastin antecedent is converted during this procedure from an inactive to an active form by an enzymatic process. Under certain conditions, clot-promoting activity of an apparently identical nature appears spontaneously in preparations of plasma thromboplastin antecedent. Activated

Hageman factor and activated plasma thromboplastin antecedent are inhibited by diisopropyl fluorophosphate and by a fraction of normal plasma.

These studies are in agreement with the hypothesis that activated Hageman factor initiates clotting by an enzymatic process. The data presented may help to explain the asymptomatic nature of Hageman trait.

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OBSERVATIONS ON DOUBLE ALBUMIN. II. A PEPTIDE DIFFERENCE BETWEEN TWO GENETICALLY DETERMINED HUMAN SERUM ALBUMINS *

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An unusual serum protein anomaly, the occurrence of two electrophoretically distinct albumins in the same serum, was recently reported by Knedel (2, 3) who observed the abnormality in eight members of two different families and termed the condition "double-albuminemia." A similar anomaly was found by Earle, Hutt, Schmid and Gitlin in 25 of 43 individuals in a single family (4, 5), and additional families with double-albuminemia have been reported by others (6–10). In all of these studies it was clear that the albumin anomaly was transmitted genetically as an autosomal characteristic, the appearance of two serum albumins in the same individual being a manifestation of the heterozygous state.

In the studies reported by Earle and co-workers, the two albumins proved to be immunochemically and ultracentrifugally indistinguishable from each other and from normal human serum albumin. Differential electrophoretic titration revealed that between pH 4.5 and 10, albumin A had a greater net negative charge than had albumin B, but at pH 3.5 and between pH 11.6 and 12.3 both albumins appeared to have the same electrophoretic mobility. The simplest explanation of the data suggested a substitution of either tyrosine, cysteine or lysine in albumin B for a carboxyl residue in albumin A. Albumin A in all respects appeared to be normal human serum albumin, while albumin B was definitely anomalous.

In the study reported here, albumins A and B were hydrolyzed enzymatically and the resultant peptides were compared by electrophoresis and chromatography. The effect of pH on the net charges of the two albumins was reinvestigated when the data suggested that lysine was the anomalous amino acid in albumin B.

METHODS

Albumins. Serum was obtained from two adults with double-albuminemia who are members of the family reported earlier by Earle and colleagues (4, 5). Each serum was fractionated according to Cohn's Method 10 as described by Lever and co-workers (11) and fraction IV + V was separated; it contained more than 90 per cent of the total albumin in the original whole serum. Approximately 80 to 85 per cent of the total protein in this fraction was albumin, and it had been ascertained earlier (5) that this fractionation method did not discriminate between albumins A and B. Albumins A and B in each fraction IV + V were separated by starch block electrophoresis (12) at pH 8.6 using veronal buffer of ionic strength 0.1; the albumins were located in the starch blocks after electrophoresis by means of filter paper prints (5) and were then eluted from the appropriate segments with water. Each albumin was subjected twice again to electrophoresis in starch blocks under the same conditions, since each, particularly albumin B, was usually contaminated with significant amounts of the other in the initial isolation. In this manner, preparations were obtained in which from 85 to more than 95 per cent of the protein was albumin B as estimated electrophoretically and immunochemically (5); the remainder of the protein in these preparations was albumin A. Over 95 per cent of the protein in the preparations of albumin A appeared to be albumin A electrophoretically and immunochemically. Three separate isolations of albumins A and B were performed with each serum.

Two normal human serum albumin preparations (kindly supplied by Dr. Walter Hughes) were obtained from pools of normal adult plasma by low temperature ethanol-water fractionation according to Method 6 of Cohn and his co-workers (13). Both albumins were crystallized (14) from fraction V, one with the aid of decanol (preparation Decanol 10) and the other as

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the mercury dimer (preparation 187-4X). Both preparations were recrystallized and were extensively characterized physicochemically and immunochemically (14–16). These albumins were dissolved in the same veronal buffer used for the electrophoretic separation of albumins A and B.

All albumins were dialyzed against distilled water for 24 to 72 hours and lyophilized prior to hydrolysis.

Enzymatic hydrolysis. Twenty to 50 mg of the lyophilized albumins was dissolved in distilled water to a concentration of 0.5 g per 100 ml and the solutions adjusted to pH 8.0 with 0.1 N NaOH. The albumins were then denatured by heating to 100° C for 15 minutes to 1 hour. The denatured albumins were hydrolyzed in the pH-stat (17) at pH 8.0 and 37° C with either chymotrypsin or trypsin using an enzyme: albumin ratio of 1: 50 by weight and using NaOH for the maintenance of pH. The enzymes were obtained from Worthington Biochemical Corp.; both were bovine in origin and crystallized twice. Digestion was allowed to proceed for 1 to 2 hours, at the end of which time hydrolysis was essentially complete. The hydrolysates were adjusted to pH 4.9 with 0.7 N HCl; at this point, small amounts of precipitate formed and these were removed by centrifugation. The supernatant hydrolysates were lyophilized and then redissolved in water to a concentration of 50 to 100 mg of original protein per ml.

Separation of peptides. The peptides present in the soluble hydrolysates were separated on sheets of Whatman no. 1 filter paper measuring 23 × 57 cm. A dry sheet was gently creased in the middle across the long axis and then laid out full length. A pencil line 7 mm in length was drawn, beginning 3 cm from one long edge and 1 cm to one side of and parallel to the middle crease. Two to 4 μl of hydrolysate, equivalent to 0.1 to 0.4 mg of albumin, was then applied to an area 1 × 7 mm on the dry paper, the application being guided by the pencil line. Application of the hydrolysate was accomplished by using a capillary pipet made by drawing out tubing having an OD of 4 mm and an ID of 1 mm. The tip of the pipet was fine enough so that the hydrolysates under gentle pressure produced a streak about 0.5 mm wide when the pipet was drawn slowly across a piece of dry filter paper. After each streak of hydrolysate was applied, the water was allowed to evaporate until the paper was *almost* but not quite dry and the hydrolysate applied again. In this way, the area of application was kept to the required size. In the waiting periods between streakings, the pipet tip occasionally plugged due to drying; this was rapidly remedied by dipping the tip into distilled water for a brief moment and then wiping the tip against an edge of filter paper.

Two papers were hung in an inverted "V" side by side over three glass rods, 3 mm in diameter, which were mounted in a rack and the papers were wet and washed at the same time with pyridine-acetic acid buffer of pH 4.29 (0.5 per cent pyridine in 0.2 M acetic acid) or pH 3.6 (1 per cent pyridine in 1.75 M acetic acid) using a 50 ml pipet beginning close to the area of application and working across and down the paper. The buffer was allowed to creep the last few millimeters to and through the application area from both sides. The rack was then immersed in a tank containing Varsol no. 1 (obtained from Esso Standard Oil Co.), buffer reservoirs at the bottom of the tank making contact with the short edges of the paper. Electrophoresis was performed at 52 v per cm of paper for 30 to 80 minutes in the same pyridine-acetic acid buffers used to wet the papers. The Varsol was cooled during electrophoresis by means of cold tap water passing through plastic coils immersed in the tank. The temperature of the tank never exceeded 32° and rarely exceeded 28° C; it was usually between 23° and 25° C.

After electrophoresis, the papers were dried at 70° C, rolled into short cylinders and subjected to ascending chromatography for 18 hours in 2,4,6-collidine: 2,6-lutidine: water, in a volume ratio of 1: 1: 1. The papers were dried at 40° to 60° C, unrolled and treated for the detection of either arginine (18), histidine (19), tryptophan (20) or tyrosine (21). Other papers were sprayed with 0.25 per cent ninhydrin in 95 per cent ethanol, and the color was developed by heating to 70° C for 15 to 30 minutes and then leaving the papers at room temperature for 1 to 3 days. The peptide patterns were photographed by reflected light, using a light green lens filter and high contrast 35 mm copying film.

Hydrolysis of peptides and separation of amino acids. Those peptides that appeared to be in different positions in the peptide patterns for the different albumins were cut out of chromatograms which were prepared as described above but sprayed instead with 0.002 per cent ninhydrin in 95 per cent ethanol. The peptides were eluted from the paper with 0.5 per cent NH_4OH or 1 per cent acetic acid, lyophilized, hydrolyzed in 0.5 ml of 5.7 N HCl at 105° C for 24 hours in a sealed tube, diluted with 5 ml distilled water, lyophilized, dissolved in 1 ml distilled water, lyophilized again and finally dissolved in 8 μl distilled water. Approximately 4 μl of the hydrolysate was applied to sheets of no. 1 Whatman filter paper measuring 20 × 20 cm and the amino acids chromatographed in two directions, first with water-saturated phenol containing quinolinol (22) and then with collidine, lutidine, and water, 1: 1: 1.

Electrophoretic mobilities. The electrophoretic mobilities of albumins A and B were determined by the free-moving boundary method employing a Perkin-Elmer instrument, model 38. For these determinations fraction IV + V (11) was used throughout. The relative positions of the two albumins on either side of their isoelectric points had been ascertained earlier (5). Electrophoresis was done over a pH range of 2.5 to 12.1 for a time period of 1 to 2.5 hours when the two albumins were readily resolved and for 3 to 4 hours when resolution was not evident. The buffers used are indicated in Figure 3.

Titration. The albumins were titrated in 0.1 N NaCl using the automatic titrator made by Radiometer Corp., Copenhagen. Sodium hydroxide or HCl was added by

means of a calibrated syringe to the protein solution kept at 25° C in a double-jacketed water-heated vessel in which the temperature did not vary more than ± 0.05° C. The amount of acid or base added, as well as the pH, was recorded automatically; pH was accurate to within 0.005 units. The molecular weight of the albumins was taken to be 65,000 (23).

RESULTS

Hydrolysis with chymotrypsin. Whereas heat-denatured human serum albumin is insoluble in water at pH 4.9, after digestion with chymotrypsin 81 to 94 per cent of the original weight of the albumins remained soluble at this pH. During hydrolysis, from 30 to 36 μmoles of NaOH per μmole of albumin of molecular weight 65,000 (23) was needed to maintain pH. In the peptide patterns obtained with these hydrolysates, approximately 55 peptides could readily be distinguished (Figures 1A and 2). Fifteen of the peptides gave strong reactions for tyrosine, while three additional peptides gave only weak reactions for tyrosine (Figure 1C). One peptide was found that contained tryptophan, ten were found that contained arginine and at least three peptides were found to contain histidine (Figure 1B).

The peptide patterns obtained for each of the albumins were reasonably reproducible whether the patterns were made with the same hydrolysate, with different hydrolysates from the same albumin preparation or with different hydrolysates from different preparations of the same albumin. Reproducible differences between the peptide patterns for albumin A and pooled normal serum albumin could not be detected. In addition, despite careful study, no peptide could be found in the peptide patterns for albumin A or in those for normal albumin that did not have a peptide in the patterns for albumin B analogous in position and type of staining reaction. In the peptide patterns for albumin B, however, one peptide was present in a position where no similar peptide was found in the patterns for normal albumin or albumin A.

The distinctive peptide of albumin B (peptide B) carried a relatively high net positive charge at pH 4.29 and pH 3.6; and indeed at pH 3.6, peptide B was one of the highest positively charged peptides in the patterns.

Tyrosine, tryptophan, histidine and arginine could not be detected in peptide B, using the specified staining reactions for these amino acids. Upon isolation of the peptide followed by acid hydrolysis, peptide B was found to yield phenylalanine, lysine, alanine, glycine, glutamic acid and aspartic acid. As controls, areas in the patterns for albumin A and normal albumin, which were equivalent in size and position to peptide B, were also eluted and hydrolyzed with acid; these did yield traces of alanine and glutamic acid.

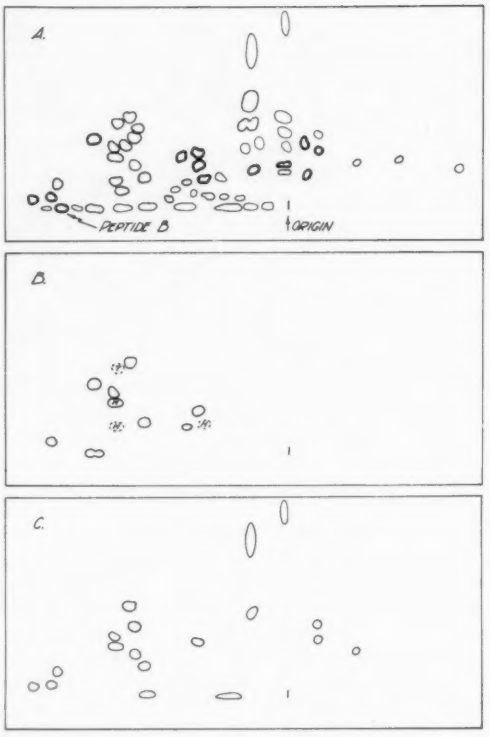

FIG. 1. COMPOSITE TRACINGS OF PEPTIDE PATTERNS OBTAINED WITH CHYMOTRYPTIC HYDROLYSATES OF ALBUMIN B. In this and in all subsequent patterns, the direction of electrophoresis is horizontal with the anode to the right of the figure, and the direction of solvent chromatography is vertical. The vertical line indicating the origin was 7 mm long. **A**. Developed with ninhydrin. **B**. Developed for arginine (solid lines), tryptophan (T) and histidine (H). **C**. Developed for tyrosine.

Hydrolysis with trypsin. From 64 to 81 per cent of the original weight of the albumins remained soluble at pH 4.9 after hydrolysis with trypsin. As with chymotrypsin, this degree of variability in hydrolysis as measured by the relative amounts of soluble and insoluble fractions

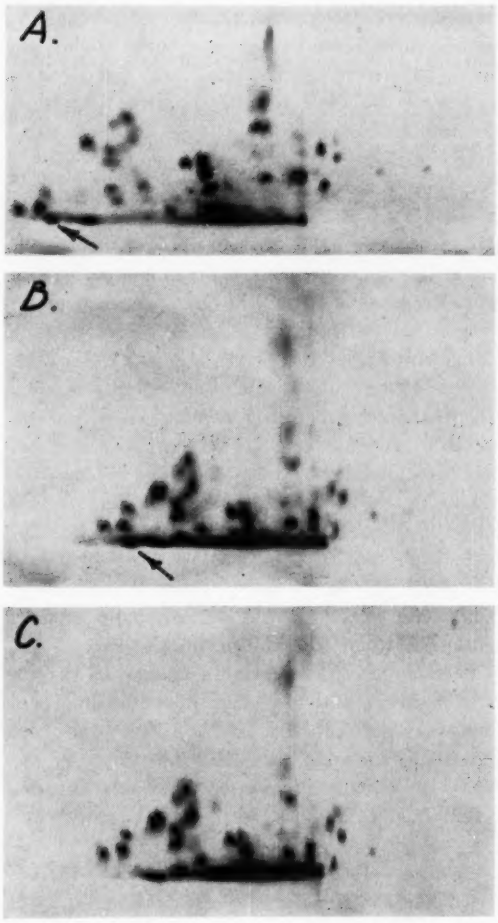

FIG. 2. PEPTIDE PATTERNS OBTAINED WITH CHYMOTRYPTIC HYDROLYSATES OF ALBUMIN A AND ALBUMIN B; DEVELOPED WITH NINHYDRIN. **A**. Albumin B; electrophoresis for 60 minutes and chromatographed with slightly more than a minimal amount of solvent. Peptide B is indicated by arrow. **B**. Albumin B, electrophoresis for 45 minutes and chromatographed in a deeper layer of solvent than **A**; peptide B is indicated by arrow. **C**. Albumin A run at the same time as **B**.

did not have a noticeable effect upon the peptide patterns obtained with the soluble portion of the hydrolysates. From 19 to 25 μmoles of NaOH per μmole of albumin was needed during digestion to maintain pH at 8.0.

Approximately 55 to 60 peptides were readily detected in the peptide patterns of these hydrolysates when stained with ninhydrin (Figures 3A and 4). Of these, 18 or 19 peptides contained arginine, at least 6 contained tyrosine and 1 contained tryptophan (Figures 3B and 3C). No reproducible differences could be found between the peptide patterns for albumin A and for the normal albumins. On the other hand, two peptides were present in the patterns for albumin B that were not apparent in the patterns for the other albumins (Figure 3A).

Elution of the two distinctive peptides of albumin B with subsequent acid hydrolysis revealed the presence of lysine, alanine, glycine, serine, glutamic acid, aspartic acid and cysteine in the more positively charged peptide, and arginine, serine and glutamic acid in the more negatively charged peptide.

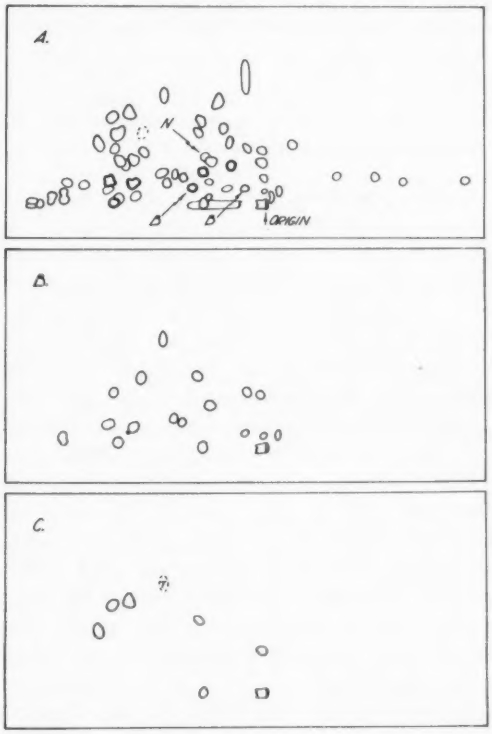

FIG. 3. COMPOSITE TRACINGS OF PEPTIDE PATTERNS OBTAINED WITH TRYPTIC HYDROLYSATES. **A**. Developed with ninhydrin. Two unique peptides of albumin B and the uncertain peptide of normal albumin and albumin A (N) are indicated by arrows. **B**. Developed for arginine. **C**. Developed for tyrosine (solid lines) and tryptophan (T).

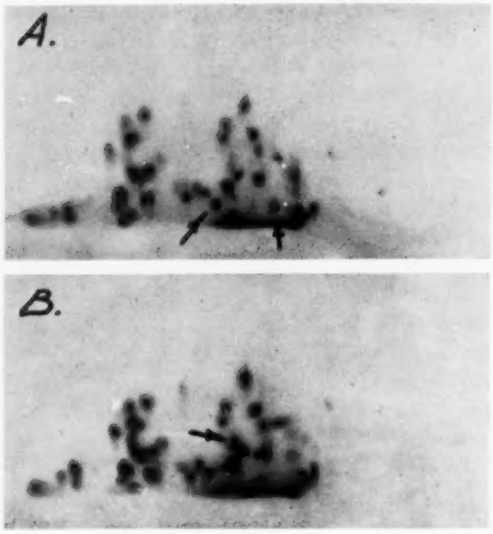

FIG. 4. TRYPTIC HYDROLYSATES, DEVELOPED WITH NINHYDRIN. **A**. Albumin B; arrows indicate unique peptides. **B**. Normal serum albumin, preparation Decanol 10, run at same time as **A**; arrow indicates the peptide of questionable uniqueness.

In the patterns for albumin A and in those for normal albumin, one peptide did appear to be present that did not seem to be present in the patterns for albumin B (Figures 3A and 4); due to close overlapping with another peptide that was present in all of the albumins, the uniqueness of this peptide could not be established either by elution or by staining reactions.

Difference in net charge between albumins A and B at various pH's. The electrophoretic mobilities of albumins A and B at various pH's are shown in Figure 5. Between pH 4.00 and 8.43, the difference in the mobilities of the two albumins was fairly constant and averaged 0.55×10^{-5} cm² per volt second; albumin A carries the more negative net charge throughout this range (5). At pH 9.96, some separation of the two albumins was noted after 4 hours, but at pH 10.8 or higher and at pH 3.5 or lower, the two albumins appeared to have the same mobilities. Interestingly, at or near pH 4.0, neither albumin A nor albumin B appeared to isomerize as does normal serum albumin; this was true whether albumins A and B were electrophoresed as a mixture or separately.

Titration of albumins A and B and normal serum albumin preparation Decanol 10 revealed no significant differences between them within the limits of experimental error, and the titration data were the same as those reported previously for preparation Decanol 10 by Tanford (15). By correlating changes in the number of moles of hydrogen ions bound per mole of albumin at different pH's with changes in the mobility of albumin A at these pH's (24), it was possible to calculate the degree of change in mobility resulting from the binding or loss of one equivalent of hydrogen ion per mole of albumin. As shown by Longsworth (24) and Schlessinger (25) for other proteins, the resulting change in mobility between pH 4.00 and pH 8.43 for albumin A was remarkably constant: 0.25×10^{-5} cm² per volt second per mole H^+ per mole albumin. Since in this pH range, the mobility of albumin B less the mobility of albumin A was 0.55×10^{-5} cm² per volt second, albumin B compared with albumin A at the same pH had a more positive net charge, equivalent to 2.2 moles H^+ per mole albumin within the range pH 4.00 to pH 8.43.

Between pH 2.5 and 3.5 the change in mobility of albumin A with a change in bound hydrogen ions was approximately 0.03×10^{-5} cm² per volt second per mole H^+ per mole albumin, and between pH 9.96 and 12.0 this value was approximately 0.04×10^{-5}.

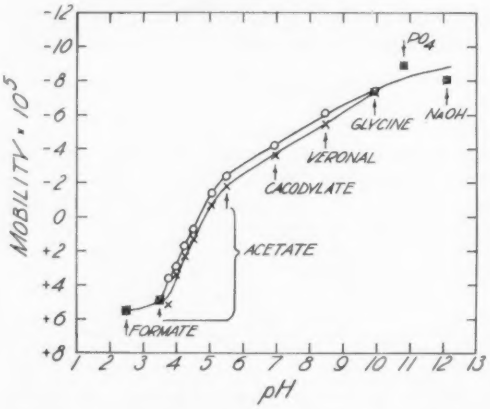

FIG. 5. THE ELECTROPHORETIC MOBILITIES OF ALBUMINS A (○) AND B (×) AT DIFFERENT PH'S. Ionic strength of formate and acetate buffers was 0.1; in the remaining buffers, ionic strength due to buffer salts was 0.02 to which was added NaCl to make the ionic strength of the final solvent 0.1.

DISCUSSION

It is apparent that a difference exists between albumin B on the one hand and albumin A and pooled normal albumin on the other in the peptides released from these proteins by enzymatic hydrolysis. The data suggest that this difference is attributable to the presence of a lysine residue in albumin B which is not present in albumin A and normal albumin. The reasons for this conclusion are as follows.

1. It had been demonstrated that, between pH 4.0 and pH 10, albumin B had a more positive net charge than albumin A (4, 5). On the basis of the charges contributed to serum albumin by the various titratable groups in different pH ranges, it was suggested that the difference in electrophoretic behavior might be attributed to a substitution of either tyrosine, lysine or cysteine in albumin B for a carboxyl residue in albumin A.

2. In peptide B, the anomalous peptide obtained by chymotryptic hydrolysis of albumin B, the only amino acid present with a positive charge at pH 4.29 or pH 3.6 was lysine; arginine and histidine were both absent. In addition, neither tyrosine nor cysteine was detected in the peptide.

3. Two peptides were found in the tryptic hydrolysates of albumin B that were not present in the tryptic hydrolysates of the other albumins. One of these peptides contained lysine and the other arginine. Since tryptic hydrolysis is limited almost exclusively to bonds involving lysine or arginine, substitution of an amino acid other than lysine or arginine in albumin B would have resulted in the presence of only one anomalous peptide rather than two, unless two amino acids were replaced. Since peptide B did contain lysine and did not contain arginine, it appears that the anomalous amino acid is lysine.

4. The more positive net charge on albumin B compared with albumin A in the pH range of 4.00 to 8.43 was equivalent to approximately two protons per molecule. This is in accord with a substitution of a single lysine residue in albumin B for a single carboxyl residue in albumin A. Since albumin appears to have but a single peptide chain, substitution of more than one lysine would have resulted in more than two anomalous peptides on tryptic hydrolysis. Admittedly, however, different parts of the albumin chain may be identical in amino acid sequence, and lysine substitutions may have occurred in identical peptide areas to yield but one anomalous peptide with chymotrypsin and two with trypsin. As will be pointed out, however, the number of peptides obtained by chymotryptic hydrolysis did approach the theoretical, suggesting that major portions of the chain at least are not identical. Another possibility is that additional anomalous peptides were present but were not observed in the peptide patterns because of overlapping with nonidentical peptides or were present in the insoluble portion of the hydrolysates.

Obviously, the case for any amino acid substitution could have been much stronger if the peptides of albumin A and normal albumin which were analogous to the anomalous peptides of albumin B had been isolated and analyzed. Unfortunately, the normal peptide equivalent of the two anomalous peptides released by trypsin could not be identified with certainty, and the nomal peptide equivalent of peptide B released by chymotrypsin could not be located at all. The reasons for the latter failure are not known, although a number of possibilities are apparent. In any event, the peptide patterns appear to support the thesis that albumin A is normal serum albumin (4, 5).

In an earlier study (5), it was pointed out that a lysine-carboxyl substitution did not explain the observation that albumins A and B appeared to have the same mobility at pH 3.5 or lower and at pH 10.0 or higher: in the low pH range, the ϵ-amino group of lysine is ionized but the carboxyl group loses its charge, whereas the reverse is true in the high pH range. Albumins A and B at these pH extremes would thus still differ by a net charge equivalent to one proton per molecule. In the present study, however, it was found that this difference in charge at these pH's resulted in a difference in mobility of only 0.03 or 0.04×10^{-5} cm^2 per volt second. Separation of the two albumins owing to this small difference in mobility would not be observed under the experimental conditions used, due to various physical disturbances, particularly those caused by the necessity for rapid continuous compensation to keep the albumins within the optical system of the equipment. At pH 9.93, 4 hours was needed to begin to see a separation of the two albumins. The failure to resolve the two albumins at the extremes of pH, therefore, was not necessarily owing to the absence

of a difference in mobility between the proteins but could instead have been due to the fact that the difference was simply too small to permit resolution.

Normal human serum albumin contains 31 phenylalanine, 17 tyrosine, 1 tryptophan, 23 arginine and 55 lysine residues (26). Since chymotrypsin hydrolyzes bonds involving phenylalanine, tyrosine or tryptophan, the total number of peptides found in the chymotryptic hydrolysates, approximately 55, agrees reasonably well with the theoretical number of 50. The number of tyrosine-containing peptides, 15 to 18, as well as the single tryptophan-containing peptide, also agrees with the amino acid composition of albumin. For trypsin, however, the total number of peptides found in the soluble hydrolysates, approximately 55, was less than the 79 expected, even though as much as 81 per cent of the albumins did appear in the soluble portion of the hydrolysates. The 18 to 19 arginine-containing peptides were also somewhat fewer than the theoretical number of 23.

It must be emphasized that, until similar studies are done with the anomalous albumins found in other families with double-albuminemia, the findings obtained with albumin B pertain only to the anomalous albumin found in the family described by Earle and colleagues (4, 5). Most likely, additional types of genetic alteration in the amino acid sequence of albumin will be found. It should also be pointed out that, although the homozygous state for a genetically determined anomalous albumin has not yet been discovered, this, too, remains a real possibility. Consequently, the term *paralbumin* has been suggested as a generic term for the anomalous albumin (5) and the term *paralbuminemia* could apply for the homozygous as well as for the heterozygous state. The terms *double-albuminemia* or *bisalbuminemia* obviously would not apply to the homozygous states.

SUMMARY

1. Two genetically determined human serum albumins were hydrolyzed with chymotrypsin and with trypsin. The resulting peptides were separated on filter paper by electrophoresis followed by chromatography. One of the two albumins, A, had the same peptide pattern as did normal human serum albumin. Chymotrypsin released one anomalous peptide from the other albumin, B, whereas trypsin yielded two anomalous peptides.

2. An electrophoretic study of the two albumins indicated that, between pH 4.00 and pH 8.43, albumin B had a more positive net charge than albumin A, equivalent to two protons per molecule.

3. The data indicate that an anomalous lysine residue is present in albumin B replacing a residue in albumin A or normal albumin which most likely contains a free carboxyl group.

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UNILATERAL HYPOVENTILATION IN MAN DURING TEMPORARY OCCLUSION OF ONE PULMONARY ARTERY *

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Occlusion of the right or left pulmonary artery forces the opposite lung to perform the total gas exchange. The alveolar ventilation of the one functioning lung must increase if it is to maintain normal arterial blood oxygen and carbon dioxide tensions (1). One would predict that total ventilation would increase about 80 to 90 per cent to achieve the necessary increase in alveolar ventilation. Söderholm, however, found only a 30 per cent increase in total ventilation when one pulmonary artery was occluded even though arterial blood oxygen saturation, carbon dioxide tension and pH did not change (2). Other investigators (3, 4) have noted even smaller changes in total ventilation during temporary unilateral pulmonary arterial occlusion (TUPAO). If tidal volume, frequency of breathing and oxygen consumption remain the same, these findings could be explained only if some or all of the ventilation of the nonperfused lung were shifted to the functioning lung.

Moore, Humphreys and Cochran (5) have shown that such a shift of ventilation did occur in most dogs when one pulmonary artery was occluded by tightening a ligature brought out through the chest wall. They attributed this shift to a loss of erectile support of the vascular bed in the lung. Venrath, Rotthoff, Valentin and Bolt (6) also reported a redistribution of ventilation in dogs when one pulmonary artery was occluded by a balloon. They believed that the shift was caused by the low CO_2 on the nonperfused side, which is consistent with Nisell's observation (7) that bronchoconstriction occurred in excised cat lungs when alveolar CO_2 was reduced. On the other hand, Folkow and Pappenheimer (8), Julian, Travis, Robin and Crump (9), and Carlens, Hanson and Nordenström (10) found no significant shift of ventilation during TUPAO.

We decided to reinvestigate the effect of unilateral cessation of pulmonary blood flow on the distribution of ventilation in dogs and man. In dogs (11) we have confirmed the findings of Nisell and of Venrath and co-workers that the low alveolar P_{CO_2} in the lung with no pulmonary arterial blood flow produces bronchoconstriction which results in redistribution of ventilation favoring the opposite lung. Our present studies indicate that such redistribution also occurs in man during TUPAO and is initiated by the same mechanism—i.e., the decrease in alveolar P_{CO_2} in the nonperfused lung.

METHODS AND MATERIALS

The plan of the investigation involved *a*) studies during and after temporary unilateral pulmonary arterial occlusion in patients before bronchospirometry and *b*) repetition of the studies in the same patients during bronchospirometry. Table I gives the physical, clinical, and pulmonary function data for the patients; all of the patients had some pulmonary disease. Control and TUPAO studies were performed 9 times in 8 patients without bronchospirometry, and once in each of the 9 patients during bronchospirometry.

Studies before bronchospirometry. With the patient supine on a fluoroscopy table, we placed a triple lumen cardiac catheter in the pulmonary artery of choice, via an antecubital vein. We then inflated a balloon near the tip of the catheter to obstruct the flow of mixed venous blood to that lung (3). The first measurements were made during the period of vascular occlusion; this allowed the control studies to follow after deflation of the balloon and eliminated any intervening period of fluoroscopy and manipulation of the catheter. We collected expired gas in a Tissot spirometer which recorded tidal volume and frequency of breathing. After three preliminary collections, we made the final collection for a 3 minute period; we drew systemic and pulmonary arterial blood samples during the second minute. We

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TABLE I

Clinical and routine pulmonary function data of ten patients studied *

Patient	Age, Sex BSA	Diagnosis	VC	MBC	RV/TLC	SB O_2	Maximal flow rates Before/After isoproterenol aerosol MEFR	MIFR	SaO_2 Breathing air	PaO_2 Breathing O_2	Dco
			% predicted		%	% N_2	L./min		%	mm Hg	% predict.
A.D.	59 M 1.82	Bilat. nodular scars, esp RUL	152		33	3.5	333/	300/	96	595	68
F.Fr.	67 M 1.68	Ca in LLL old tbc RUL	117	119	33	4.0	273/375	186/214	91		64
J.J.	33 M 1.86	Bilat. midlung tbc infilt.	131	120	13	3.5	500/	375/	96	650	111
D.K.	23 M 1.71	Emphysema; bilat. bronchiect.	79	35	41	11.0	75/83	255/280	97		76
L.M.	63 M 1.48	Ca in RUL; inactive bilat. apical tbc	82	64	48	6.5	83/88	115/115	88	525	34
L.P.	50 M 1.91	Oat cell Ca in RLL	139		23	4.0	250/	214/	93		172
N.P.	60 M 1.73	LLL bullae	104	134	34	3.5	430/	375/	96		80
E.T.	52 M 1.74	Ca in RLL; inactive bilat. apical tbc	96	50		5.0	123/150	123/136	85	659	
E.D.	62 M 1.70	Ca + abscess in SS, LLL	105	81	46	5.0	57/117	96/110	89		
A.G.	71 M 1.98	RUL tumor	101		54	5.0	214/200	200/200	95	510	

* Abbreviations: VC = vital capacity; MBC = maximal breathing capacity; RV/TLC = residual volume per total lung capacity; SB O_2 = rise N_2 concentration between 750 and 1,250 ml expired after a single breath of pure O_2 (1); MEFR and MIFR = maximal expiratory and inspiratory flow rates (1); SaO_2 = % arterial oxygen saturation; PaO_2 = arterial oxygen tension; Dco = breath-holding diffusing capacity for carbon monoxide at rest; RUL = right upper lobe; SS, LLL = superior segment of left lower lobe; Ca = carcinoma.

measured the O_2 and CO_2 concentrations of expired gas (using the Scholander apparatus) and of blood (using the Van Slyke apparatus). We determined systemic arterial O_2 and CO_2 tensions with special electrodes (12). We calculated O_2 uptake, CO_2 elimination, respiratory quotient, cardiac output, physiological dead space and alveolar ventilation.

We calculated anatomic dead space during and after TUPAO from continuous recordings of expired CO_2 concentration and flow (13); CO_2 concentration was obtained with the Beckman Spinco apparatus and flow with a Fleisch pneumotachygraph. The lag of the CO_2 meter was measured at the time each patient was studied; it averaged 0.1 second.

In order to determine whether venous to arterial shunts were present during the vascular occlusion, we gave two patients (O.B. and A.G.) pure oxygen to breathe for 20 minutes before we deflated the balloon; they continued to breathe oxygen during and after balloon deflation. We measured the oxygen tension of arterial blood (12) just before deflation and 1 minute afterwards. Finally, to determine whether shunts were due to easily reversible atelectasis, we asked the patients to take several deep breaths and repeated the measurement of arterial blood P_{O_2}.

Studies during bronchospirometry. At the completion of the previous studies, we injected intravenously 0.75 to 1.0 mg of atropine sulfate and 75 to 100 mg of pentobarbital sodium as premedication for bronchospirometry. The patient then sat up and we anesthetized his airway with 1 per cent tetracaine, and passed a Carlens catheter (French 41) by indirect laryngoscopy. With the patient again supine and breathing air, we collected expired gas from each lung simultaneously in meteorologic balloons. We noted the time of collection and the respiratory rate so that we could calculate mean tidal volume. We determined the oxygen uptake, carbon dioxide output, minute and tidal volumes of each lung. Under fluoroscopic control we then reoccluded the pulmonary artery to one lung and repeated the above studies.

We then measured the anatomic dead space in each lung in sequence, starting with the perfused lung. During each determination, a sample of systemic arterial blood was drawn for measurement of CO_2 tension. Low tidal volumes, especially on the side of the pulmonary arterial occlusion, made measurements of the anatomic dead space difficult, but they were nevertheless reproducible within a limit of 5 ml.[1] While monitoring CO_2 and air flow of the lung with a blocked pulmonary artery, we then deflated the occluding balloon. Figure 1 shows an example of the increase in alveolar (end tidal) CO_2 and flow.[2]

[1] Calculated anatomic dead space volumes were corrected for 15 ml of apparatus dead space (catheter lumen and tubing) so that they represent volumes "below the carina."

[2] Low end tidal CO_2 values were useful as a criterion of the unilateral blockade of perfusion. However, oxygen

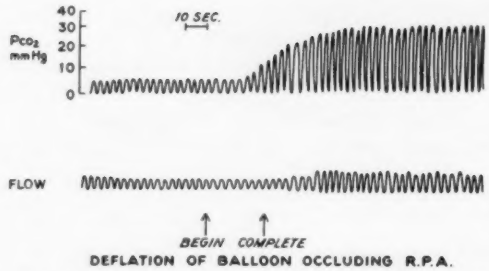

FIG. 1. INSPIRED AND EXPIRED P_{CO_2} AND FLOW TRACINGS IN PATIENT L.P. MONITORED AT THE EXTERNAL ORIFICE OF THE RIGHT LUMEN OF THE BRONCHOSPIROMETRIC CATHETER BEFORE, DURING AND AFTER DEFLATION OF THE BALLOON BLOCKING THE RIGHT PULMONARY ARTERY.

We again measured anatomic dead space on each side. The time between the dead space measurements of the right and left lung during either the control period of unilateral pulmonary arterial occlusion was less than 5 minutes.

In the last two patients (E.T. and E.P.) we tested the effect of administering 6 per cent CO_2 (in air or oxygen) to the unperfused lung to determine the effect of changing concentrations of CO_2 on the distribution of tidal volume between the two lungs.

uptake data showed that the occlusion was not always complete, even when an allowance was made for an enlarged bronchial collateral flow of blood and the mild hypoxemia associated with the patient's disease and the nature of the procedure (see Discussion).

RESULTS

Table II shows the results of nine studies in eight patients with the airway undivided. Associated with the occlusion, there was no significant increase in total ventilation. The unchanged arterial CO_2 tension suggests that alveolar ventilation remained normal even though there was no increase in total ventilation. Mean values for alveolar ventilation (total minus physiologic dead space ventilation) were 4.21 L per minute after, and 4.10 during the occlusion, a decrease of 3 per cent (not statistically significant). The mean oxygen uptake and carbon dioxide output each decreased 8 per cent (the differences again were not statistically significant) and the respiratory quotient stayed approximately the same. The physiologic dead space increased slightly while the mean anatomic dead space decreased slightly. The mean values for both the arteriovenous oxygen difference and the cardiac index decreased slightly but not to a statistically significant degree.

TABLE II

Studies "at the mouthpiece" (undivided airway) after and during temporary unilateral pulmonary arterial occlusion *

Patient	PA occluded side		$\dot{V}_E$	f	V_T	P_{aCO_2}	Physiol. V_D	$\dot{V}_A$	Anat. V_D	$\dot{V}_{O_2}$	$\dot{V}_{CO_2}$	RQ	AVD	CI
		min	*L/min*	*per min*	*ml*	*mm Hg*	*ml*	*L/min*	*ml*	*ml/min*	*ml/min*		*ml/L*	*L/min/m²*
A.D.	O		7.10	16	444	39	210	3.74		216	176	0.814	40	2.98
	R	15	6.84	11	622	41	313	3.40		205	168	0.818	43	2.63
F.Fr.	O		9.23	22	419	46	272	3.25	148	208	171	0.821	46	2.69
	L	15	9.92	22	451	45	301	3.30	130	227	175	0.770	42	3.21
J.J.	O		8.86	12	738	39	286	5.43	170	297	236	0.796	58	2.76
	R	20	10.48	14	749	40	376	5.22	140	300	236	0.787	45	3.60
D.K.	O		9.75	20	487	36	226	5.23		273	215	0.788	40	4.00
	R	20	11.61	21	552	34	256	6.23		282	240	0.851	42	3.93
	O		10.35	22	470	36	247	4.92		256	207	0.808	52	2.92
	R	15	11.30	20	565	34	307	5.16		264	198	0.750	38	3.95
L.M.	O		9.53	22	433	39	264	3.72	90	209	167	0.799	41	3.44
	R	75	10.05	24	418	37	264	3.71	100	174	158	0.910	42	2.81
L.P.	O		9.20	14	657	40	338	4.47	90	276	208	0.754	38	3.66
	R	15	6.06	14	433	42	239	2.71	80	178	134	0.798	33	2.54
N.P.	O		7.44	13	572	40			105					
	L	86	7.78	12	648	41			100					
E.T.	O		10.89	26	419	33	252			242	205	0.848	35	3.97
	R	20	9.60	24	400	35	250	3.60		206	167	0.815	39	3.03
Mean control			9.15	19	516	39	262	4.21	121	265	212	0.861	44	3.31
TUPAO		31	9.30	18	536	39	288	4.10	110	246	197	0.870	41	3.21

* Abbreviations: $\dot{V}_E$ = total ventilation; f = respirations per minute; V_T = tidal volume; P_{aCO_2} = arterial CO_2 tension; Physiol. V_D = physiologic dead space uncorrected for 100 ml apparatus dead space; $\dot{V}_A$ = alveolar ventilation; Anat. V_D = anatomic dead space; $\dot{V}_{O_2}$ = oxygen uptake; $\dot{V}_{CO_2}$ = carbon dioxide output; RQ = respiratory quotient; AVD = arteriovenous O_2 content difference; CI = cardiac index.

TABLE III

Ventilation studies during temporary unilateral pulmonary arterial occlusion and bronchospirometry *

Patient	Duration of PAO	Insp. gas	$\dot{V}_E$	f	Lung	V_T	% of total	$\dot{V}_{O_2}$ Bilat.	% of total	$\dot{V}_{CO_2}$ Bilat.	% of total	P_{aCO_2}	PET_{CO_2}	Anat. V_D
	min		*L/min*	*per min*		*ml*		*ml/min*				*mm Hg*		*ml*
A.D.	O	Air	6.50	10	R	297	48	302	50			40	35	
					L	323	52		50				35	
	R15	Air	10.94	13	R	280	35	299	2	218	5	40	4	
					L	527	65		98		95		39	
F.Fr.	O	Air	5.24	22	R	176	41	169	42			46	32	35
					L	253	59		58				28	45
	L10	Air	8.91	22	R	287	62	206	100	146	95	41	34	45
					L	156	38		0		5		3	25
J.J.	O	Air	17.99	15	R	489	44	324	50	318	48	29	22	45
					L	620	56				52		22	45
	R5	Air	21.59	18	R	519	46	444	16	401	21	34	4	15
					L	590	54		84		79		28	15
D.K.	O	Air	12.20	20	R	282	49	276	39	252	39	33	29	
					L	286	51		61		61		32	
	R5	Air	12.18	23	R	128	27	352	8	251	9	33	6	
					L	372	73		92		91		33	
L.M.†	O	Air	16.35	14	R	473	42	311	38	278	32	45	31	21
					L	665	58		62		68		26	14
	R10	Air	4.51	15	R	60	20	215	9	107	8	50	16	19
					L	240	80		91		92		39	13
L.P.	O	Air	10.03	20	R	155	38	443	56			43	29	20
					L	255	62		44				33	20
	R24	Air	9.31	19	R	40	9	284	0	238	1	45	4	20
					L	408	91		100		99		33	15
N.P.	O	Air	6.45	14	R	235	55	248	54			42	13	30
					L	195	45		46				12	25
	L13	Air	6.39	16	R	240	65	249	82			42	17	20
					L	130	35		18				9	15
E.T.	O	Air	14.52	21	R	219	36	294	32	206	33	32	20	
					L	395	64		68		67		20	
	R5	Air	18.94	26	R	198	28	349	13	302	15		9	
					L	499	72		87		85		21	
	R5	6% CO_2	15.83	26	R	207	36	295	1	221	0		36	
		Air			L	373	64		99		100		23	
E.P.	O	Air	10.05	12	R	485	60	333	72	263	66		33	
					L	322	40		28		34		30	
	L25	Air	12.65	16	R	611	80	269	96	235	95	38	30	
					L	151	20		4		5		4	
	L35	O_2	14.80	20	R	385	54						28	
		6% CO_2			L	325	46						43	
Mean	Control		10.37	17	Ips	277	45	299	44	260	37	38	26	31
					Con	337	55		56		63		27	
TUPAO			12.61	19	Ips	200	29	274	8	256	9	40	11	19
					Con	490	71		92		91		29	

* Abbreviations as in Table II plus: % =per cent of the bilateral function performed by that lung; PET_{CO_2} =end tidal CO_2 tension; Ips =ipsilateral lung, of which pulmonary artery was or was subsequently blocked; Con =contralateral lung. Anatomic dead space values are corrected for 15 ml apparatus dead space (bronchospirometric tubing) so that the volumes are "below the carina."

† Morphine sulfate, 5 mg, given i.v. because of paroxysmal coughing, so decreased the ventilation that only the R:L distribution values are included in the mean data for the group.

Table III shows the results of TUPAO during bronchospirometry in nine patients. The total ventilation (mean) increased 22 per cent; however, the ventilation of the lung with the occluded pulmonary artery was only 29 per cent of the total as compared with a control value of 44 per cent. The anatomic dead space of that lung also decreased. When 6 per cent CO_2 in air or oxygen was breathed by the unperfused lung (Patients E.T. and E.P.), the fraction of the total ventilation of that lung returned to control values.

DISCUSSION

Unilateral pulmonary arterial obstruction in a normal individual must create a considerable increase in physiologic dead space, if ventilation of alveoli on this side continues. Under these conditions, total ventilation should increase about 80 per cent to maintain normal O_2 and CO_2 tensions in systemic arterial blood. Nevertheless, previous investigators (2–4) have not found an increase in total ventilation of the magnitude predicted, and our study confirms this.

There are several possible ways of explaining how arterial blood O_2 and CO_2 tensions can remain normal during TUPAO with little or no increase in over-all ventilation.

1. The pulmonary circulation may have been occluded by disease *before* the balloon occlusion. This was not true in our patients; bronchospirometric studies showed that the O_2 consumption of the experimental lung averaged 44 per cent of the total for the two lungs in the control period, and in only one patient (E.P.) was it less than 30 per cent of the total.

2. TUPAO may be accompanied by a decrease in body metabolism which occurs either coincidentally (change in environment) or by some reflex action. A decrease in O_2 consumption may be responsible in part in Patients L.P. (Table II) and L.M. (Table III), but the mean values of O_2 consumption were only 8 per cent lower than during the control period, which could not explain the data observed.

3. Breathing may change to a slower and deeper pattern so that alveolar ventilation may increase, although over-all minute ventilation does not. This mechanism may afford a partial explanation in Patient A.D. (Table II), but in the other patients there was either little change in respiratory frequency or there was an increase.

4. Anatomic dead space may decrease. The mean value decreased from 121 to 110 ml during TUPAO. This change is too small to explain the data observed.

5. Redistribution of inspired air may occur so that a large fraction of it enters perfused alveoli and little enters the nonperfused lung.

We believe that the major factor in the maintenance of normal arterial blood O_2 and CO_2 tensions during TUPAO is a redistribution of inspired air. The data obtained during bronchospirometry show unequivocally that the percentage of inspired air directed to the nonperfused lung decreases (mean decrease from 45 to 29 per cent) and that to the other lung increases (mean increase from 55 to 71 per cent). There is a suggestion that the shift is more effective when the airway is not divided, since the change in total ventilation was less than that noted during bronchospirometry. Atropine, used as premedication for bronchospirometry, may act to prevent bronchoconstriction, since it is known to increase the anatomic dead space (14). However, in our animal studies, atropine did not alter the ventilatory shift associated with the unilateral occlusion (11). Topical anesthesia may also play a role. It is also possible that the procedure of bronchospirometry leads to an increase in sympathetic nervous system activity and that this tends to decrease the response. We have been able to block the response completely by administering isoproterenol as an aerosol to the side with the arterial occlusion or by infusing epinephrine into the main pulmonary artery in animals (11). A mechanism such as this may have been responsible for the negative results in some previous studies in dogs (9) and patients (10). Patient J.J. failed to show any decrease in his ipsilateral tidal volume during TUPAO; his mean systemic arterial pressure rose gradually from a normal level to 150 mm Hg during bronchospirometry and TUPAO.

The mechanism responsible for the redistribution of inspired air in man appears to be the same as in the dog: TUPAO leads to decreased elimination of CO_2 ipsilaterally and to a decrease in alveolar CO_2 tension; this in turn leads to bronchoconstriction. Addition of CO_2 to the gas inspired

by the experimental lung, so that its alveolar P_{CO_2} remains normal despite TUPAO, prevents the redistribution (6, 7, 11). Pressure upon the bronchus by the inflated balloon in the pulmonary artery cannot be responsible, because bronchograms in two subjects could not demonstrate any encroachment upon the airway near the balloon (Figure 2). The low pressure distal to the occluding balloon in the pulmonary artery does not initiate the redistribution, as was postulated by Moore and colleagues (5), because the shift fails to occur when the balloon is inflated if the inspired gas contains 6 per cent CO_2 (11).

Airway-narrowing during TUPAO can proceed to the point of atelectasis. When we deflated the occluding balloon in two patients breathing oxygen, the systemic arterial blood O_2 tension decreased abruptly, indicating that there was a pulmonary arterial to venous shunt on that side. Since the arterial P_{O_2} rose to or toward control values after deep breathing, we suspect that the shunt was through atelectatic regions which could be opened up by voluntary hyperinflation (Table IV).

Unilateral bronchoconstriction may also be responsible for delayed exhalation by the involved lung. This was suggested by the alteration in the "alveolar plateau" (when expired CO_2 was monitored at high paper speed) during unilateral pulmonary arterial occlusion in some of the patients. As can be seen in Figure 3, the normal slight upward slope of the alveolar plateau was changed to an irregular downward sloping pattern, somewhat similar to that described by West and Hugh-Jones in studies in animals (15).

TABLE IV

The effect of deflation of pulmonary arterial (PA) balloon and of deep breathing on P_{aO_2} (patients breathing O_2 throughout)

Patient	PAO	Arterial O_2 tension (mm Hg)		
		Before deflation of PA balloon	Immediately after deflation of PA balloon	After deep breathing
O.B.	L	520	420	520
A.G.	R	590	400	510

We were not always able to eliminate the oxygen uptake on the side of the TUPAO. Despite roentgenographic confirmation of the position of the balloon, the blockade was probably incomplete in two cases (J.J. and N.P., Table III). The mean residual oxygen uptake of the "unperfused" lung may be in part attributable to an enlarged collateral circulation bringing slightly desaturated bronchial arterial blood into the pulmonary capillary bed. The arterial oxygen tensions were in the range of 70 to 80 mm Hg; however, no correlation was present between the size of the oxygen uptake on the side of the occluded artery and the

FIG. 2. LEFT-SIDED BRONCHOGRAM VIA THE LEFT LUMEN OF THE BRONCHOSPIROMETRIC CATHETER IN PATIENT F.FR. DURING TEMPORARY OCCLUSION OF HIS LEFT PULMONARY ARTERY.

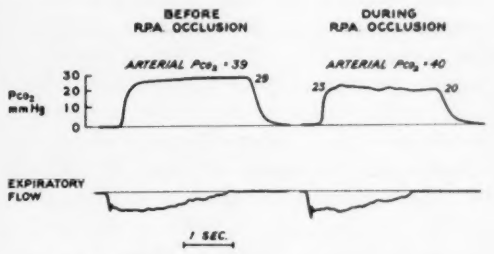

FIG. 3. CO_2 AND AIR FLOW TRACINGS AT THE MOUTHPIECE (UNDIVIDED AIRWAY) BEFORE AND DURING RIGHT PULMONARY ARTERIAL OCCLUSION IN PATIENT J.J. Note the changes in the "alveolar plateau" as well as the arterial-alveolar CO_2 tension differences associated with the occlusion.

arterial oxygen tension or saturation before vascular occlusion when the airway was undivided. The CO_2 output of the "unperfused" lung was consistently greater (mean of 9 per cent of the bilateral CO_2 output) than the contribution of that lung to the total oxygen uptake. The high respiratory quotient resulting is explainable on the basis of the high CO_2 gradient and ready diffusibility of this gas from the bronchial collateral (systemic arterial) blood to the alveoli of the lung whose supply of mixed venous blood is blocked.

SUMMARY

1. In eight patients with chronic lung disease, studied during and after temporary unilateral pulmonary arterial occlusion (TUPAO) in order to determine the ventilatory response to one-sided obstruction of the flow of mixed venous blood: *a*) On the average, the total ventilation was unchanged. *b*) At the same time, the mean arterial P_{CO_2} did not rise, indicating that alveolar hypoventilation had not occurred. *c*) The physiologic dead space increased less than might have been expected considering that 45 per cent of the total ventilation and oxygen uptake was originally performed by the lung whose pulmonary artery was later blocked. *d*) In several cases the "alveolar plateau" of CO_2 became irregular during the occlusion and sloped downward; this was interpreted as delayed emptying of the lung whose CO_2 output was curtailed from lack of perfusion.

2. In nine patients, studied by bronchospirometry and unilateral pulmonary arterial occlusion: *a*) After obstructing the flow of blood to one lung the tidal volume of that lung decreased so that its contribution to the total ventilation diminished from a mean of 45 to 29 per cent during the occlusion. *b*) During unilateral pulmonary arterial occlusion the anatomic dead space in that lung (below the carina) decreased from a mean of 31 ml before, to 19 ml during occlusion. *c*) Although the total ventilation increased by a mean of 22 per cent, under these circumstances the shift in the distribution of ventilation allowed the contralateral lung to increase its ventilation by a mean value of 63 per cent (5.73 L per minute before, 9.32 during occlusion); the normal arterial P_{CO_2} figures (mean of 38 before, 40 mm Hg during) attest to the magnitude of this shift. *d*) Allowing the "unperfused" lung to breathe 6 per cent CO_2 in two cases resulted in a return to the preocclusion values for distribution of inspired gas to the right and left lungs.

3. We suggest that the immediate reduction of ventilation in one lung following the occlusion of its pulmonary artery is due to bronchoconstriction which occurs in response to local airway hypocapnia and leads to regional atelectasis.

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THE RELATIONSHIP OF RENAL OXYGEN CONSUMPTION TO RENAL FUNCTION AND WEIGHT IN INDIVIDUALS WITH NORMAL AND DISEASED KIDNEYS *

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In previous studies of renal oxygen consumption in man the results have been expressed in the following terms: *a*) the total oxygen consumed per two kidneys per minute ($T_{Q_{O_2}}$) (1); *b*) the quantity of oxygen utilized per 100 g per minute based on the measurement of $T_{Q_{O_2}}$ and an assumed weight of 300 g per two kidneys (1); *c*) the $T_{Q_{O_2}}$ as related to total renal blood flow (TRBF) and glomerular filtration rate (GFR) (2). In the later instance Cargill and Hickam (2) concluded that $T_{Q_{O_2}}$ was best correlated with the GFR. Bradley and Halperin (3), on the other hand, suggested that the $T_{Q_{O_2}}$ of the kidney was probably related to the "tubular functional mass" as determined by the tubular maximal secretory capacity for sodium para-aminohippurate (Tm_{PAH}). However, as noted by Smith (4), no data have been forthcoming with regard to the latter relationship.

Therefore, the present paper is an attempt: *1*) to provide data in which the relationship between the total renal oxygen consumption and the Tm_{PAH} is defined in normal subjects and in patients with kidney disease; *2*) to demonstrate the limitations of such a ratio ($T_{Q_{O_2}}/Tm_{PAH}$); and *3*) to suggest that in future studies $T_{Q_{O_2}}$ be considered alone and/or that oxygen consumption be determined as Q_{O_2} per 100 g per minute.

MATERIAL AND METHODS

Five "normal" [1] subjects, 5 patients with essential hypertension (grade 2-3), 5 with diabetic nephropathy, 5 with chronic pyelonephritis, and 5 with chronic glomerulonephritis were selected from the wards of the University Hospitals between 1952 and 1958. In addition, one each of the following types of patients was studied as a "control": "juvenile" diabetes mellitus without clinical evidence of retinopathy or nephropathy; essential hypertension before and after the administration of oral glucose and intravenous insulin. In all cases the diagnoses were made on clinical rather than morphological grounds.

All patients, with the exception of those with diabetes mellitus, were studied in a fasting state. The exceptions were given their breakfast as a liquid glucose equivalent [2] along with their daily dose of insulin 1 hour prior to the start of the test. Evidence that such a procedure did not appear to influence the results is presented in the Discussion.

Simultaneous determinations of glomerular filtration rate, total renal plasma flow (TRPF), TRBF, arterio-renal venous oxygen difference ($A\text{-}RV_{O_2}$), $T_{Q_{O_2}}$, renal blood flow per 100 g per minute (RBF), renal oxygen consumption per 100 g per minute (Q_{O_2}), and *in vivo* renal weight (R_{wt}) were made, utilizing right renal vein catheterization and techniques previously described for this laboratory (5, 6).

The results, contained in the tables, represent the average of three 15-minute periods. They are uncorrected for surface area since the relationships to be discussed did not lend themselves to correction and/or resulted in cancellation of this correction by virtue of being present in both the numerator and the denominator. Furthermore, the mean values for surface area of the 5 groups did not differ significantly from one another per meter ^{2} ("normals," 1.80; essential hypertension, 1.81; chronic glomerulonephritis, 1.80; diabetic nephropathy, 1.77; chronic pyelonephritis, 1.82).

Statistical analyses were performed, utilizing the standard *t* test (7).

RESULTS

The results are presented in Tables I–III.

Table I contains the individual data. Table II represents the mean values for each function of

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[1] "Normal" subjects were individuals diagnosed as having "psychoneurotic reaction" (000–X0Y).

[2] The liquid glucose equivalent was calculated as 100 per cent of the carbohydrate, 58 per cent of the protein, and 10 per cent of the fat which would have been consumed at breakfast.

TABLE I

Individual renal and metabolic data *

Patient	GFR	TRBF	RBF	R_{wt}	Tm_{PAH}	$\frac{Tm_{PAH}}{R_{wt}}$	$\frac{GFR}{Tm_{PAH}}$	$\frac{GFR}{R_{wt}}$	$\frac{TRBF}{Tm_{PAH}}$	A-RV_{O_2}	$T_{Q_{O_2}}$	Q_{O_2}	$\frac{T_{Q_{O_2}}}{Tm_{PAH}}$	$\frac{T_{Q_{O_2}}}{GFR}$
	ml/min	*ml/min*	*ml/100 g/min*	*g*	*mg/min*	*mg/min/g*	*ml/mg*	*ml/min/g*	*ml/mg*	*vol%*	*ml/2 kidneys/min*	*ml/100 g/min*	*ml/mg*	*ml/ml*
Normal														
M.F.	133	1,800	528	342	98	0.29	1.36	0.390	18.4	1.3	23.4	6.9	0.237	0.176
N.G.	91	1,440	300	480	88	0.18	1.03	0.190	16.3	1.4	20.2	4.2	0.236	0.222
K.T.	86	1,255	342	368	62	0.17	1.39	0.243	20.2	1.2	15.1	4.1	0.172	0.176
K.L.	81	935	302	310	88	0.28	0.91	0.261	10.5	1.8	16.8	5.4	0.191	0.207
R.S.	104	1,505	494	305	86	0.28	1.21	0.341	19.7	1.4	20.5	6.8	0.237	0.197
Essential hypertension														
E.K.	51	710	410	173	60	0.35	0.85	0.295	11.8	1.7	12.1	7.0	0.203	0.237
L.A.	87	1,215	398	305	85	0.28	1.03	0.285	14.3	1.2	14.6	4.8	0.172	0.168
K.L.	90	1,070	303	353	88	0.25	1.03	0.254	12.2	1.8	19.3	5.5	0.219	0.215
S.R.	62	630	336	188	69	0.37	0.90	0.329	9.2	2.1	13.2	7.1	0.192	0.213
D.N.	57	655	426	151	63	0.41	0.91	0.370	10.4	2.0	13.1	8.5	0.218	0.203
Chronic glomerulonephritis														
M.H.	52	675	307	220	47	0.21	1.11	0.236	14.4	1.5	10.1	4.6	0.215	0.193
D.K.	44	755	265	285	55	0.19	0.80	0.154	13.7	1.0	7.6	2.7	0.139	0.173
C.B.	87	1,085	279	389	101	0.26	0.86	0.224	10.8	1.8	19.5	5.0	0.197	0.224
D.A.	71	1,110	249	446	76	0.17	0.93	0.159	14.6	1.2	13.4	3.0	0.176	0.189
P.M.	15	350	300	117	19	0.16	0.79	0.128	18.4	1.8	6.3	5.4	0.331	0.420
Diabetic nephropathy														
A.K.	30	455	160	284	21	0.07	1.43	0.106	21.3	1.2	5.6	2.0	0.266	0.187
W.F.	19	465	144	323	25	0.08	0.76	0.059	18.6	1.4	6.5	2.0	0.260	0.342
E.McM.	61	790	208	378	57	0.15	1.07	0.162	13.9	2.1	16.8	4.4	0.293	0.275
H.B.	44	665	233	285	35	0.12	1.25	0.154	19.0	1.4	9.3	3.3	0.265	0.212
M.W.	36	640	298	230	32	0.14	0.12	0.157	19.4	1.3	8.3	3.9	0.269	0.230
Chronic pyelonephritis														
P.S.	30	390	157	248	30	0.12	1.00	0.121	13.0	2.1	8.2	3.2	0.273	0.272
W.H.	11	210	134	157	11	0.07	1.00	0.070	12.2	1.3	2.7	1.7	0.245	0.245
E.W.	41	610	189	322	47	0.15	0.87	0.127	13.0	1.5	9.2	2.8	0.195	0.224
R.G.	21	300	121	247	19	0.08	1.11	0.085	15.8	1.9	5.7	2.3	0.300	0.272
L.E.	27	210	63	331	18	0.05	1.05	0.081	11.7	4.1	8.6	2.6	0.486	0.318

* See text for abbreviations.

any given group, the standard errors of such means, as well as the level of statistical significance (p) when the mean of a given function in a particular disease group is compared with the mean of the corresponding function of the "normal" individuals. The material contained in Table III has been reserved for the Discussion.

Normal. The results obtained in such individuals were in accord with those previously reported from this (5, 6) and other laboratories (4). The values for GFR may be questioned as being normal. However, "normal" mean values given by other investigators, using the inulin clearance technique, with PAH at low plasma levels, have ranged between 114 and 140 ml per minute with standard deviations of ± 4.4 to ± 40 ml per minute (4). Also, the GFR's presented in Table I were obtained at the same time as the determinations of Tm_{PAH}. The latter procedure, based on 30 different investigations in as many patients, produced a decrease in GFR of 19 per cent (8) over those obtained before PAH saturation. This is in accord with previous observations (4).

Essential hypertension. A comparison of the data of these patients with those obtained in "normal" subjects shows that GFR ($p < 0.05$), TRBF ($p < 0.02$), and the $T_{Q_{O_2}}$ ($p < 0.05$) were significantly reduced. In contrast, the A-RV_{O_2} ($p > 0.2$), the RBF per 100 g per minute ($p > 0.7$), as well as the renal Q_{O_2} per 100 g per minute ($p > 0.3$) were not significantly different from normal. The decline in TRBF and $T_{Q_{O_2}}$, as compared with the lack of change in these functions when expressed on a unit weight basis, is probably related to the significant reduction in renal weight ($p < 0.05$). The mean value for Tm_{PAH} was 11 mg per minute less than the mean value for this function in normal subjects, but this difference was not statistically significant ($p > 0.12$).

The ratio of the $T_{Q_{O_2}}$ to the Tm_{PAH} or to the GFR remained unaltered ($p > 0.3$ in both instances). The significant decrease in TRBF/

TABLE II

Summary and statistical analysis of Table I

Type	No. of pts.	GFR	TRBF	RBF	R_{wt}	T_{mPAH}	$\frac{T_{mPAH}}{R_{wt}}$	$\frac{GFR}{T_{mPAH}}$	$\frac{GFR}{R_{wt}}$	$\frac{TRBF}{T_{mPAH}}$	A-RVO_2	T_{QO_2}	QO_2	$\frac{T_{QO_2}}{T_{mPAH}}$	$\frac{T_{QO_2}}{GFR}$
		ml/min	*ml/min*	*ml/100 g/min*	*g*	*mg/min*	*mg/min/g*	*ml/mg*	*ml/min/g*	*ml/mg*	*vol%*	*ml/2 kidneys /min*	*ml/100 g/min*	*ml/mg*	*ml/ml*
"Normal"	5														
Mean		100	1,390	393	361	84	0.24	1.19	0.289	17.0	1.4	19.2	5.5	0.215	0.196
SE*		±9.1	±142	±49	±32	±5.8	±0.027	±0.09	±0.032	±1.8	±0.10	±1.5	±0.60	±0.012	±0.009
Essential hypertension	5														
Mean		69	855	375	235	73	0.33	0.95	0.306	11.6	1.7	14.6	6.6	0.201	0.213
SE		±8.0	±119	±24	±39	±5.4	±0.029	±0.04	±0.020	+0.9	±0.16	±1.3	±0.65	±0.007	±0.012
p†		<0.05	<0.02	>0.7	<0.05	>0.2	<0.05	<0.05	>0.7	<0.05	>0.2	<0.05	>0.3	>0.3	>0.3
Chronic glomerulonephritis	5														
Mean		54	795	280	291	60	0.20	0.89	0.180	14.4	1.5	11.4	4.1	0.212	0.240
SE		±12.2	±141	±11	±58	±14	±0.018	±0.06	±0.021	±1.2	+0.16	±1.9	±0.55	±0.034	±0.046
p		<0.02	<0.02	<0.05	>0.3	>0.1	>0.2	<0.05	<0.05	>0.3	>0.6	<0.02	>0.01	>0.9	>0.4
Diabetic nephropathy	5														
Mean		38	605	209	304	34	0.11	1.13	0.128	18.4	1.5	9.3	3.1	0.271	0.249
SE		±7.0	±63	±28	±25	±6.2	±0.015	±0.11	±0.020	±1.2	±0.16	±2.0	±0.46	±0.006	±0.025
p		<0.001	<0.001	<0.009	<0.2	<0.001	>0.01	>0.7	<0.01	>0.7	>0.6	<0.01	<0.02	<0.01	<0.05
Chronic pyelonephritis	5														
Mean		26	345	133	261	25	0.09	1.10	0.097	13.1	2.2	6.9	2.5	0.300	0.266
SE		±4.9	±74	±21	±31	±6.2	±0.018	±0.11	±0.012	±0.8	±0.50	±1.6	±0.25	±0.055	±0.015
p		<0.001	<0.001	<0.001	<0.05	<0.001	<0.01	>0.6	<0.001	<0.05	>0.1	<0.001	<0.01	<0.1	<0.01

* SE = standard error of the mean.
† p = Level of statistical significance when mean of group is compared with mean of "normals." For other abbreviations see text.

TABLE III

Control studies

Patient no.	GFR	TRBF	Tm_{PAH}	$\frac{GFR}{Tm_{PAH}}$	$\frac{TRBF}{Tm_{PAH}}$	A-RVO_2	TQ_{O_2}	$\frac{TQ_{O_2}}{Tm_{PAH}}$	$\frac{TQ_{O_2}}{GFR}$
	ml/min	*ml/min*	*mg/min*	*ml/min*	*ml/min*	*vol%*	*ml/2 kidneys /min*	*ml/mg*	*ml/ml*
1. Diabetes mellitus without nephropathy	65	865	72	0.90	12.0	1.8	15.6	0.216	0.240
2. Essential hypertension C*	58	740	49	1.14	15.1	1.4	10.4	0.213	0.178
E†	53	650	49	1.08	13.3	1.5	9.8	0.200	0.185

* Control, fasting.
† Thirty to 60 minutes after 0.1 U insulin/kg i.v., plus 100 g glucose p.o.

Tm_{PAH} ($p < 0.05$) and the constancy of the GFR/R_{wt} ($p > 0.7$) were expected, in view of the fact that the pathological changes in such kidneys are primarily of a vascular nature. In contrast, the significant reduction in GFR/Tm_{PAH} ($p < 0.05$), and the increase in Tm_{PAH}/R_{wt} ($p < 0.05$), were unexpected findings. A possible explanation for these observations is presented in a later section.

Chronic glomerulonephritis. As in patients with essential hypertension, the GFR ($p < 0.02$) and TRBF ($p < 0.02$) were significantly decreased. However, when these functions were related to the Tm_{PAH}, a significant reduction was noted in GFR/Tm_{PAH} ($p < 0.05$), while TRBF/Tm_{PAH} remained unchanged ($p > 0.3$). Furthermore, there was a fall in GFR/R_{wt} ($p < 0.05$). Such findings indicated the primary glomerular involvement in this entity. The absence of a change in TRBF/Tm_{PAH} ($p > 0.3$), despite significant reductions in TRBF ($p < 0.02$) as well as RBF per 100 g per minute ($p < 0.05$), is interpreted to mean that the fall in renal blood flow was proportionate to the progressive renal damage in this disease.

Tm_{PAH}/R_{wt} remained unchanged ($p > 0.1$) while all of the other estimations were similar to those found in the individuals with essential hypertension.

Diabetic nephropathy. The features which distinguished this group of patients from "normal," as compared with the groups with essential hypertension and glomerulonephritis, were the significant reductions noted in Tm_{PAH} ($p < 0.001$), Tm_{PAH}/R_{wt} ($p < 0.01$), Q_{O_2} per 100 g per minute ($p < 0.02$) and the significant increases in TQ_{O_2}/Tm_{PAH} ($p < 0.01$) and TQ_{O_2}/GFR ($p < 0.05$). Furthermore, GFR/Tm_{PAH} and TRBF/Tm_{PAH}, which heretofore, either alone or in combination were significantly altered, remained unchanged in these individuals.

Chronic pyelonephritis. Patients in this group, with two exceptions, were similar from both functional and metabolic standpoints to the patients with diabetic nephropathy. First, although the mean value for TQ_{O_2}/Tm_{PAH} was increased to an even greater extent (0.300 ml per mg) than that observed in the patients with diabetic nephropathy (0.271 ml per mg) it was nevertheless not found to be statistically different from normal, due to the variability of the individuals within the group. Secondly, TQ_{O_2}/GFR was significantly increased ($p < 0.01$).

DISCUSSION

It is obvious that the renal TQ_{O_2} represents the sum of the oxygen consumed by the individual parts of the kidney. However, in the past, efforts have been made to relate the former quantity of oxygen to one functional measurement [e.g., TRBF (9, 10), GFR (2)]. Therefore, since it was conceivable that TQ_{O_2} might be primarily related to TRBF, to the "tubular functional mass" or to glomerular function and, since all of these functions have not heretofore been measured simultaneously in patients with "normal" and diseased kidneys (4), the present study was undertaken in an effort to test whether such a primary relationship existed.

Total renal blood flow. Van Slyke, Rhoads, Hiller and Alving (9), as well as others (10),

initially concluded that the total renal oxygen consumption was related to the total renal blood flow. Subsequent studies by Clark and Barker (1) and Barker and Crosley and co-workers (11–13), utilizing measures which alter total renal blood flow, showed that such was not the case in normal human subjects or in patients with renal disease. Thus the arterio-renal venous oxygen difference could indeed narrow or widen in response to change in blood flow, thereby maintaining an unchanged oxygen consumption. Furthermore, since $T_{Q_{O_2}}$ is derived as the product of TRBF and the arterio-renal venous oxygen difference, one would predict a high correlation between TRBF and $T_{Q_{O_2}}$ on a purely mathematical basis. In addition, it follows that the narrower the range of the arterio-renal venous oxygen differences the higher the correlation. In the present study this range was so narrow that there was no significant difference between the arterio-renal venous oxygen differences of the diseased groups and those of the "normals"; hence a correlation between TRBF and $T_{Q_{O_2}}$ was not considered applicable.

"Tubular functional mass." Although it had been suggested by Bradley and Halperin (3) that the most likely relationship between the $T_{Q_{O_2}}$ and the renal functional elements was between $T_{Q_{O_2}}$ and the "tubular functional mass" ($T_{Q_{O_2}}/Tm_{PAH}$), no data on this ratio have been forthcoming until the present study (4). Support for such a relationship is the finding here that the correlation between these two factors is highly significant ($R = +0.923$, $p < 0.001$).

Glomerular filtration rate. As previously noted, Cargill and Hickam (2) reported a positive correlation between the total oxygen consumption of the kidney and the glomerular filtration rate. On the surface, such findings pose the problem as to why oxygen consumption should be related to a function whose primary energy source is that of the net hydrostatic pressure of the blood. However, recent evidence by Dubach and Recant (14) lend support to such a relationship, since their *in vitro* investigations of individual glomeruli attribute important oxidative functions to this structure. The present data are also in accord with the concept that total oxygen consumption is related to glomerular activity, since the correlation coefficient between these two functions, utilizing all of the patients in the five groups, was $R = +0.955$ ($p < 0.001$).

In view of the highly significant correlation coefficients for both GFR and Tm_{PAH} as related to $T_{Q_{O_2}}$ it appears that one explanation for such data is that, in renal disease, there is a symmetrical destruction of both glomeruli and tubules and that associated with the loss of such nephrons there is a proportionate reduction in $T_{Q_{O_2}}$. Such a statement would be in agreement with the findings of Bricker, Morrin and Kime in unilaterally induced renal disease in dogs, which appear to demonstrate proportionate declines in glomeruli and tubules (15). An alternative explanation would be that suggested by Cargill and Hickam (2), that tubular activity is largely governed by the quantity of glomerular filtrate and thus a close correlation between GFR, Tm_{PAH} and $T_{Q_{O_2}}$ might be expected.

Finally, the unexpected findings of the increased $T_{Q_{O_2}}/Tm_{PAH}$ and $T_{Q_{O_2}}/GFR$ and the significant decreases in Tm_{PAH}/R_{wt} in patients with diabetic nephropathy and chronic pyelonephritis, as well as the decreased GFR/Tm_{PAH} in essential hypertension, must be considered. No definitive explanation is forthcoming for any of these results.

However, in the case of the increased $T_{Q_{O_2}}/Tm_{PAH}$ and $T_{Q_{O_2}}/GFR$ in patients with diabetic nephropathy and chronic pyelonephritis, these changes do not appear to be due to: *a*) the diabetic state per se, since they occurred in patients without diabetes (e.g., chronic pyelonephritis and Patient P.M. with glomerulonephritis); *b*) the presence of insulin and/or the glucose-equivalent breakfast, since a control diabetic patient (Table III, no. 1) and a nondiabetic patient (no. 2) given a "glucose-insulin" tolerance test did not show elevations of these ratios; *c*) the oxygen consumption of nonexcretory renal tissue (e.g., scar tissue), since the Q_{O_2} of such tissue is lower than that of excretory renal tissue (16). In view of the significant decreases in Q_{O_2} per 100 g per minute associated with the increased $T_{Q_{O_2}}/Tm_{PAH}$ and $T_{Q_{O_2}}/GFR$, one can only speculate that the latter findings may be related to the fact that a total quantity (oxygen) was related to only a part (Tm_{PAH} and/or GFR) of the total metabolizing tissue of the kidney (Tm_{PAH} and/or GFR plus scar and exudative tissue). In a similar

fashion, one can only hypothesize that the significant depression in Tm_{PAH}/R_{wt}, in these cases, may be due to the fact that a significant portion of the kidney weight was composed of nonexcretory renal tissue (scar tissue). If so, the ratio Tm_{PAH}/R_{wt} may serve as an index of renal "scarring."

The observation of a significant decrease in GFR/Tm_{PAH} in patients with essential hypertension has not been the usual finding in other series (4). Such data may be fortuitous, since the number of patients was small and the significance was borderline ($p < 0.05$). However, a recent report by Baldwin and associates (17) in patients with essential hypertension revealed a decline in the GFR/Tm_{PAH} in the majority of the cases. Furthermore, White, Sambhi and Grollman have reported such findings in experimentally induced hypertension in rats (18).

SUMMARY AND CONCLUSIONS

1. A number of observations was made in individuals with "normal" and diseased kidneys in an effort to determine whether any single functional component [total renal blood flow (TRBF), glomerular filtration rate (GFR), "tubular functional mass" (Tm_{PAH})] was a primary contributor to the total oxygen consumed per two kidneys per minute ($T_{Q_{O_2}}$). None could be found.

2. Since in certain disease categories (diabetic nephropathy and chronic pyelonephritis) $T_{Q_{O_2}}/Tm_{PAH}$ or $T_{Q_{O_2}}/GFR$ was increased, while at the same time $T_{Q_{O_2}}$ and renal oxygen consumption per 100 g per minute (Q_{O_2}) were decreased, it is suggested that in future studies $T_{Q_{O_2}}$ be considered alone or, if a base reference is desired, that renal oxygen consumption be determined in terms of Q_{O_2} per 100 g per minute.

3. Tm_{PAH}/R_{wt} was significantly reduced in patients with chronic pyelonephritis and diabetic nephropathy, while the GFR/Tm_{PAH} was reduced in essential hypertension. No definitive explanation is forthcoming for such findings.

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SIGNIFICANCE OF URINARY HYDROXYPROLINE IN MAN

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Since nearly all of the hydroxyproline of the body is found in collagen, it has been suggested (1, 2) that the urinary excretion of this imino acid may be an important index of collagen metabolism. The origin of urinary hydroxyproline, however, is not definitely established. The isotopic studies of Stetten (3) in rats indirectly suggested that most of the free and peptide hydroxyproline in the body arises from the breakdown of collagen, since she found that hydroxyproline-N^{15} was not significantly incorporated into collagen. Ziff, Kibrick, Dresner and Gribetz (1), on the other hand, observed an increased excretion of hydroxyproline when it was added to the diet of human subjects, in the form of gelatin. Although Ziff and co-workers did not emphasize this point, their results suggested the possibility that under normal conditions much of urinary hydroxyproline may originate from dietary proteins.

The present study was undertaken to investigate further the factors that influence hydroxyproline excretion in man. Although ingestion of large amounts of gelatin was found to increase urinary hydroxyproline, its urinary excretion did not decrease when hydroxyproline was eliminated from the normal diet, or even if subjects were placed on a protein-free diet. No diurnal variation in urinary hydroxyproline was found, and hydration or dehydration did not alter excretion. Ingestion of large amounts of hydroxyproline as the free imino acid resulted in an increased excretion of free, but not of bound hydroxyproline, the peptide form which normally accounts for nearly all of the hydroxyproline in urine.

These findings indicate that the daily excretion of hydroxyproline is largely independent of factors that influence the excretion of other amino acids, and they support the suggestion that urinary hydroxyproline arises from the breakdown of body collagen. The results are consistent with the hypothesis that the large hydroxyproline excretions found in children (1) and in some cases of Marfan's syndrome (2) reflect a rapid rate of collagen degradation.

An incidental discovery in the study was that the increase in urinary hydroxyproline after ingestion of gelatin represents an increased excretion of hydroxyproline peptides. This appears to be the first demonstration that significant amounts of peptides can be excreted following ingestion of a protein.

MATERIALS AND METHODS

The 8 subjects utilized in the study were hospitalized for periods of 3 to 12 weeks; 3 were patients with Marfan's syndrome, 2 of whom were previously shown to have elevated excretions of hydroxyproline (2). The remaining 5 were normal adult volunteers—2 males and 3 females.

Procedures. Urine specimens were collected daily under 30 ml toluene and stored at 0° C for periods up to 1 year. It was found that, in the absence of preservative, hydroxyproline was converted from the peptide-bound to the free form in normal urine, and that hydroxyproline disappeared rapidly in urines containing large amounts of the free imino acid. For the first 5 days after admission, the subjects were placed on an *ad lib.* diet, but thereafter they were maintained on a diet low in hydroxyproline. In some subjects, after 3 weeks had elapsed on this diet, oral supplements of various amino acids and proteins were administered on alternate days. Urine from the previous day was used as a control in each case. Each dietary supplement was given in 250 ml of orange juice at the beginning of a urine collection period, and the subjects were kept in a fasting state for 8 hours before and 4 hours after each administration. In a few instances the unpleasant taste of the supplements necessitated administration by gastric tube.

Diets and dietary supplements. The low hydroxyproline diet was similar to the one described by Ziff and associates (1) and provided 1,800 to 2,300 calories per day with 70 to 90 g protein, 200 to 300 g carbohydrate and 80 to 90 g fat, but no gelatin-containing foods such as meat, fish, Jell-o, soft candy or ice cream. It was estimated to contain less than 100 mg hydroxyproline per day. The low protein diet was prepared daily by mixing 150 g of sucrose and 150 g of melted butter into 300 ml of warm water and then adding the mixture to a thin paste made from 20 g of flour and water. Since the flour was the only nitrogen-containing constituent, and

since flour contains negligible amounts of hydroxyproline (4), the diet was essentially free of hydroxyproline. It provided 1,750 calories per day distributed as 120 g fat, 165 g carbohydrate and 3 g protein.

The various amino acids were purchased from Nutritional Biochemicals Corp. All were L isomers except that DL forms of serine, threonine and valine were used in preparation of the mixture of amino acids found in gelatin (5). The gelatin was obtained from a commercial source. The casein hydrolysate (Casamino Acids) was from Difco Laboratories, and analysis showed that it contained less than 0.04 per cent hydroxyproline.

Urinary assays. Initially, urinary hydroxyproline was measured by the method of Mitoma and co-workers (6), but subsequent determinations were by a new procedure described elsewhere (7). From 0.1 to 4 ml of untreated urine containing 5 to 50 μg of hydroxyproline was used to measure free hydroxyproline. An equivalent amount of hydrolyzed urine was used to determine total hydroxyproline, which was considered to be the sum of free and peptide-bound hydroxyproline.

Urinary total nitrogen was measured by a micro-Kjeldahl method (8), and α-amino nitrogen by the method of Troll and Cannan (9).

RESULTS

The normal subjects on the study excreted from 16 to 34 mg of hydroxyproline per day, and the three patients with Marfan's syndrome excreted from 38 to 90 mg per day when on an *ad lib.* diet (Table I). In agreement with the results of Ziff and colleagues (1), the free hydroxyproline was less than 3 per cent of the total excretion and in most instances was too small to measure accurately (less than 0.5 μg per ml of urine).

Effect of restricting dietary hydroxyproline and total protein intake. After 5 days on an *ad lib.* diet, three normal subjects and three patients with Marfan's syndrome were placed on the low hydroxyproline diet for periods of 3 to 6 weeks. At the end of this time there was no significant change in their hydroxyproline excretions (Table I). The moderate increase in the case of K.L. may be related to a weight loss of 3 kg sustained over the 3 week period (10); he found the low hydroxyproline diet unpalatable. Similarly, the decrease of hydroxyproline excretion in the case of G.H. may be related to a weight gain of 4 kg over the 3 week period.

Four of the subjects from this group were placed on the low protein diet for 3 days after completing 3 to 6 weeks on the low hydroxyproline diet. There was no further change in their hydroxyproline excretions (Table I, column 3). Two normal subjects (A.F. and R.A.) were placed on the low protein diet after 3 weeks' observation on *ad lib.* diet. As shown in Table II, the hydroxyproline excretion remained constant

TABLE I

Failure of low hydroxyproline diet and low protein diet to decrease hydroxyproline excretion *

Subject	*Ad lib.* diet		Low hydroxyproline diet 3–6 weeks		Low protein diet 3 days	
	Free hydroxy.	Bound hydroxy.	Free hydroxy.	Bound hydroxy.	Free hydroxy.	Bound hydroxy.
	mg./day		*mg./day*		*mg./day*	
J.R.	<1.0	27 (23–31)†	<1.0	30 (24–36)	<1.0	37 (28–42)
K.L.	<1.0	32 (30–34)	<1.0	46 (44–51)	<1.0	46 (43–48)
G.H.	<1.0	21 (16–29)	<1.0	16 (13–22)	<1.0	14 (13–15)
R.F.‡	<1.0	62 (46–88)	<1.0	61 (42–92)	<1.0	64 (59–69)
R.B.‡	<1.0	39 (38–40)	<1.0	38 (33–43)		
B.F.‡	<1.0	77 (63–91)	<1.0	79 (75–84)		

* Values for free and bound hydroxyproline are means of five 24-hour excretions on the *ad lib.* or low hydroxyproline diet and the means of three 24-hour excretions on the low protein diet.

† Figures in parentheses are the range of bound hydroxyproline excretions.

‡ Marfan's syndrome.

TABLE II

Effect of low protein diet on hydroxyproline, total nitrogen, and α-amino nitrogen excretion

Diet	Total hydroxy.		Total nitrogen		α-Amino nitrogen	
	J.B.	R.A.	J.B.	R.A.	J.B.	R.A.
	mg/day		*g/day*		*g/day*	
Ad lib.	31.7	32.7	6.51	14.88	1.11	3.48
	25.2	27.1	8.96	13.38	2.25	3.38
	27.4	29.2	9.35	14.65	1.48	3.24
Low hydroxy-proline	23.9	33.1	6.58	10.56	2.16	3.66
	20.2	26.2	6.22	11.93	2.07	3.00
	27.6	39.9	10.24	11.80	1.21	3.92
Low protein	27.4	26.1	5.16	8.88	1.08	1.41
	26.1	29.0	4.43	5.33	1.09	1.85
	28.2	31.3	3.86	4.67	0.47	2.32

even though the low protein diet produced a decrease in the excretion of both urinary total nitrogen and α-amino nitrogen.

Diurnal pattern of hydroxyproline excretion. To investigate any possible diurnal variations in the rate of hydroxyproline excretion, urine was collected by 6-hour periods on 2 consecutive days from two normal subjects and one patient with Marfan's syndrome, while all three subjects were on the low hydroxyproline diet (Table III). There was no consistent pattern in the amount of hydroxyproline excreted, and there were no apparent changes with daytime activities or with meals (served at 8 a.m., 1 p.m. and 5 p.m.).

Effect of hydration and dehydration. To determine the influence of moderate water diuresis on the renal excretion of hydroxyproline, two normal subjects were placed on the low hydroxyproline diet and their urine collected from 8 a.m. to 4 p.m. for 9 consecutive days. On 3 control days the subjects were allowed free access to water. During 3 days of hydration they were given an additional 500 ml water at midnight, 500 ml at the start of the collection, and 500 ml at 12 noon. On the final 3 days of the study the daily total fluid intake was limited to 500 ml. As shown in Table IV, there was no consistent change in hydroxyproline excretion with threefold changes in urine volumes.

TABLE III

Diurnal pattern of hydroxyproline excretion (mg/6 hours)

	6 a.m.–12 m.	12 m.–6 p.m.	6 p.m.–12 mid.	12 mid.–6 a.m.
A.F.	10.6	6.7	8.4	8.2
	10.0	12.9	7.4	4.8
R.A.	8.5	5.8	12.2	7.1
	7.9	14.7	7.0	7.8
R.F.*	19.7	16.6	22.2	22.0
	17.1	17.8	33.6	19.3

* Marfan's syndrome.

Effect of hydroxyproline and gelatin ingestion. Ziff and associates (1) found that no increase in total urinary hydroxyproline occurred when the low hydroxyproline diet was supplemented with 4 g of hydroxy-L-proline. However, a four- to fivefold increase was observed when gelatin containing an equivalent amount of hydroxyproline was administered.

In repeating the experiments, we were also unable to demonstrate any significant increase in total urinary hydroxyproline after administration of 4 g of hydroxy-L-proline (Figure 1). There was, however, a significant increase in free hydroxyproline (from less than 1 mg per day to 5 to 16 mg) in four subjects tested. A fifth subject, K.L. (Figure 2) showed no change. When the diet was supplemented with 12 g of hydroxy-L-proline, there were dramatic increases in the urinary free hydroxyproline in all eight subjects tested; the increases shown in Figure 1 are typical. It is important to note that, within the limits of the analyses, there were no changes in the excretion of bound hydroxyproline. Furthermore, there was no increase in bound hydroxyproline when the same subjects were given a synthetic mixture of the amino acids found in gelatin (Figure 1, column 4). In contrast, when 28 g of gelatin (containing 4 g of hydroxyproline) was given, the increase in urinary hydroxyproline was al-

TABLE IV

Failure of hydration and dehydration to influence hydroxyproline excretion (see text)

Period	Subject A.F.		Subject J.B.	
	Urine vol	Hydroxy.	Urine vol	Hydroxy.
	ml	*mg/8 hrs*	*ml*	*mg/8 hrs*
Control	985	9.7	780	8.4
	620	9.4	920	6.5
	1,000	10.1	840	6.6
Hydration	1,470	9.5	1,580	10.5
	1,560	12.5	1,390	8.7
	1,620	10.0	1,650	9.0
Dehydration	250	11.5	380	10.5
	500	10.2	410	12.5
	490	9.2	475	7.7

most entirely in the peptide-bound fraction (Figure 1, column 5). Five g of gelatin did not change free or bound hydroxyproline in the same subjects. Two normal subjects (J. B. and G. H.) were given 100 g of gelatin in a single dose. Their free hydroxyproline excretion rose from less than 1 mg per day to 200 and 300 mg, respectively. Simultaneously, their bound hydroxyproline rose from mean values of 25 and 39 mg to 356 and 427 mg.

Throughout this study there was no consistent difference between the response of normal subjects and patients with Marfan's syndrome to the feeding of gelatin or free hydroxyproline.

Effect of other amino acids on excretion of hydroxyproline. In examining the results shown in Figure 1, it is seen that more free hydroxyproline was excreted by all four subjects when 4 g of hydroxy-L-proline was given with a mixture of the amino acids found in gelatin than when 4 g of hydroxy-L-proline was ingested alone. To explore the significance of this finding, hydroxy-L-proline was given to four subjects with hydrolyzed casein and with individual amino acids (Figure 2). All four excreted more hydroxyproline when the imino acid was given with the hydrolyzed casein than when it was administered alone, but the change in one instance (K.L.) was small. When hydroxyproline was given with proline or glutamic acid, a similar increase in the amount ex-

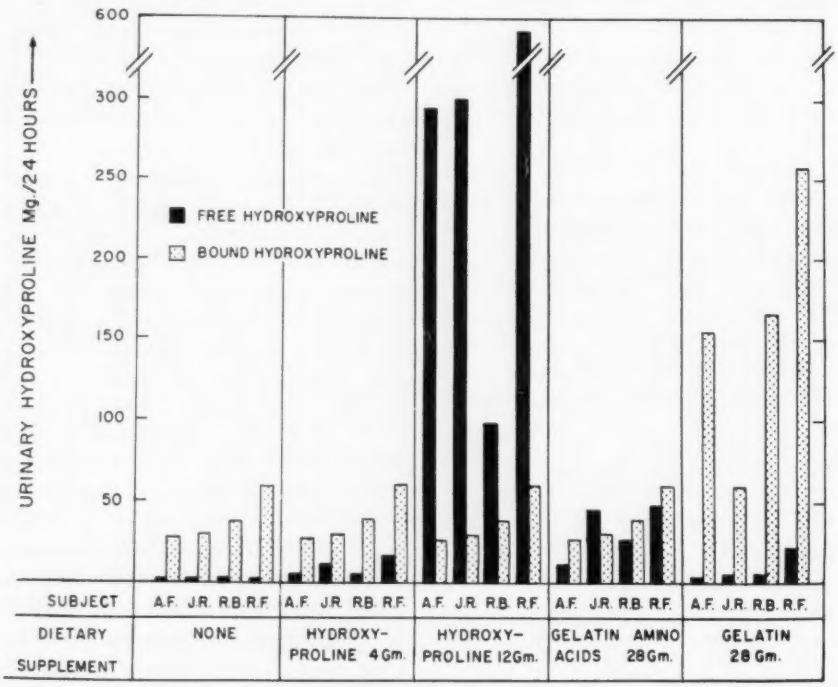

FIG. 1. EFFECT OF HYDROXYPROLINE AND GELATIN INGESTION ON THE EXCRETION OF FREE AND BOUND HYDROXYPROLINE. "Gelatin amino acids" was a prepared mixture of the amino acids found in gelatin (5). Patients R.B. and R.F. had Marfan's syndrome.

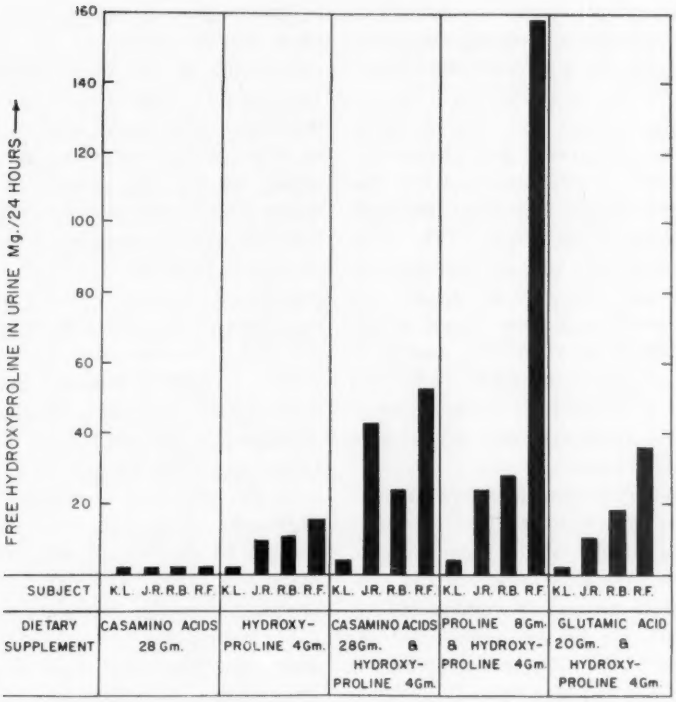

FIG. 2. EFFECT OF HYDROXYPROLINE AND AMINO ACID MIXTURES ON THE EXCRETION OF FREE AND BOUND HYDROXYPROLINE. Patients R.B. and R.F. had Marfan's syndrome.

creted occurred in three of the four subjects (Figure 2). The administration of proline, glutamic acid or hydrolyzed casein alone did not alter hydroxyproline excretion.

DISCUSSION

Currently available evidence indicates that bound hydroxyproline, the form which predominates in human urine, consists of hydroxyproline peptides. Westall (11) described a peptide in human urine containing equal amounts of proline and hydroxyproline, and Mechanic, Skupp, Safier and Kibrick (12) recently determined the amino acid composition of two hydroxyproline peptides in the urine of a patient with rheumatoid arthritis.

Since collagen contains nearly all of the hydroxyproline in the body, the hydroxyproline found in urine could arise from three possible sources: from dietary hydroxyproline, from precursors involved in the synthesis of collagen, or from peptides released by the breakdown of collagen.

Although ingestion of large amounts of gelatin results in increased hydroxyproline excretion (1), dietary hydroxyproline is not an important source of urinary hydroxyproline. Ziff and associates (1) found that patients on diets containing less than 100 mg of hydroxyproline excreted 20 to 40 mg of the imino acid per day. Similarly, it was found here (Table I) that there was no significant decrease in urinary hydroxyproline as subjects were changed from a normal to a low hydroxyproline diet, and finally to a diet that was virtually free of hydroxyproline.

At the present time there is little evidence to suggest that urinary hydroxyproline comes from hydroxyproline peptides which are precursors in collagen synthesis. Ziff and associates (1) considered the possibility that the increased excretion of hydroxyproline peptides in children is the result of an increased pool of hydroxyproline pep-

tide "available for collagen synthesis." According to current concepts, however, exchangeable pools of peptides do not play an important role in protein synthesis, and the synthesis of hydroxyproline peptides has not yet been demonstrated. The failure of the low protein diet to decrease hydroxyproline excretion (Tables I and II) also makes it unlikely that urinary hydroxyproline peptides represent collagen precursors. The subjects on the low protein diet received less than 0.5 g of nitrogen per day and were in negative nitrogen balance even though their caloric intake was normal. Under these conditions there is a general decrease of protein synthesis (13), and one would expect a decrease of hydroxyproline excretion if urinary hydroxyproline arises from pools of collagen precursors.

The results presented here therefore support the hypothesis that urinary hydroxyproline arises primarily from the breakdown of body collagen. It is of interest that the composition of the peptides studied by Mechanic and co-workers (12) is not consistent with the known amino acid sequences of collagen (14). The reason for this is not clear, and a full explanation will probably have to wait for a complete understanding of the structure of collagen. The peptides may be altered after release from collagen, or possibly may represent ester-bonded fragments which result from transverse cleavage of the 3-stranded helical structure postulated for collagen (15, 16).

If it is accepted that urinary hydroxyproline arises primarily from body collagen, then at least three factors might be expected to influence the amount of hydroxyproline excreted: the rate of plasma clearance by the kidneys, the rate at which hydroxyproline peptides are degraded further to carbon dioxide and urea, and the rate of collagen breakdown.

There is no evidence to suggest that variations in renal clearance are important in determining the amount of hydroxyproline excreted. Children excrete three to five times as much hydroxyproline as do adults, even though total amino acid excretion is less in children (1). Similarly, some patients with Marfan's syndrome excrete three to four times as much hydroxyproline as do controls, but their total amino acid excretion is normal (2). The excretion of free hydroxyproline was potentiated by the simultaneous feeding of proline or glutamic acid (Figure 2), probably because of competition for the same reabsorption mechanism in the renal tubules. Kamin and Handler (17) reported similar results with the infusion of other amino acids in dogs. There may be other explanations for our results here, but at any rate the large amounts of amino acids necessary to demonstrate the effect make it unlikely that this mechanism normally has much influence on urinary hydroxyproline. It was not possible to test directly for any effect which other peptides may have on the excretion of bound hydroxyproline, but it was shown that endogenous hydroxyproline excretion was not increased following meals (Table III) and that the excretion of hydroxyproline was not related to the rate of urine formation (Table IV).

At the moment it is impossible to estimate the degree to which hydroxyproline peptides from collagen breakdown are metabolized to carbon dioxide and urea. Although these are the major products from the degradation of other proteins (18), several observations suggest that the degradation of collagen hydroxyproline is not complete. The studies of Stetten and Schoenheimer (19) indicate that hydroxyproline does not share in the same "dynamic equilibrium" as other amino acids. The results with the low protein diet (Table II) support this conclusion and show that hydroxyproline excretion is not influenced by the same factors that influence the excretion of other amino acids. Since it has been shown that only minimal amounts of peptides are absorbed during protein digestion (20), the increase of bound hydroxyproline excretion after the ingestion of gelatin (Figure 1) raises the possibility that all of the hydroxyproline peptides absorbed were excreted in the urine. These exogenous peptides may be altered before excretion (12), but the results suggest that they are metabolized slowly, if at all. The same may be true for endogenous ones, but the situation may vary in different tissues (21).

If a relatively small part of the hydroxyproline from collagen degradation is metabolized to carbon dioxide and urea, then hydroxyproline excretion should be directly related to the rate of collagen breakdown. Studies on collagen metabolism in animals are consistent with this hypothesis, since hydroxyproline excretion decreases with age (1) and the turnover rate of collagen is much

slower in old animals than in young (22). As discussed by Ziff and associates (1), these differences may be related to the decrease of the various soluble collagens in old animals (23) and suggest that the amount of hydroxyproline found in urine directly reflects the proportion of body collagen in a soluble form. The increased hydroxyproline excretion in some cases of Marfan's syndrome may therefore indicate increased amounts of soluble collagen and a rapid rate of collagen breakdown in this condition. We are currently undertaking experiments to test this hypothesis further.

SUMMARY

Several factors that might affect the urinary excretion of hydroxyproline were studied in five normal subjects and in three patients with Marfan's syndrome.

Urinary excretion of hydroxyproline did not decrease when subjects were changed from a normal to a low hydroxyproline diet, or to an isocaloric low protein diet. No diurnal variation in urinary hydroxyproline was found, and hydration or dehydration did not alter excretion.

Ingestion of hydroxyproline as the free imino acid resulted in an increased excretion of free hydroxyproline, but no change in the excretion of bound hydroxyproline, the peptide form which normally accounts for nearly all of the hydroxyproline in urine. Ingestion of large amounts of hydroxyproline in the form of gelatin, however, increased the excretion of hydroxyproline peptides.

The findings suggest that the bound hydroxyproline normally found in urine arises from collagen breakdown and that the amount excreted is an index of the rate of degradation of this protein.

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THE METABOLISM OF CREATINE-1-C^{14} BY MICE WITH HEREDITARY MUSCULAR DYSTROPHY *

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Creatinuria, diminished urinary creatinine excretion and low concentrations of creatine in skeletal muscle are characteristic findings in progressive muscular dystrophy in man (1). These abnormalities of creatine metabolism are sometimes thought to be nonspecific changes secondary to a smaller muscle mass (2), but this has not been proven; and, because of the probable importance of phosphocreatine to energy metabolism in skeletal muscle (3), it is possible that an abnormality of creatine metabolism would contribute to the weakness if not to the actual damage of muscle fibers in muscular dystrophy. It is therefore important to establish the nature of the defect in creatine metabolism in muscular dystrophy.

Since the hereditary myopathy of mice described by Michelson, Russell and Harman (4) resembles progressive muscular dystrophy, an investigation of creatine metabolism in these mice was undertaken. It was found that the muscle creatine of the dystrophic mouse has a shortened turnover time [1] which suggests that the low concentration of creatine in the skeletal muscle in this condition is due to an impaired ability to retain creatine.

METHODS

Strain 129 dystrophic mice and their normal litter mates, approximately 8 weeks of age and of both sexes, were obtained from the Roscoe B. Jackson Memorial Laboratory. They were given a purified diet consisting of casein,[2] 30 g; sucrose, 59 g; hydrogenated vegetable fat (Crisco), 3 g; cod liver oil, 3 g; salt mixture (6), 5 g; choline chloride, 100 mg; DL-α-tocopheryl acetate, 15 mg; thiamine hydrochloride, 1 mg; riboflavin, 1 mg; nicotinamide, 4 mg; inositol, 20 mg; calcium pantothenate 2 mg; pyridoxine hydrochloride, 1 mg; biotin, 10 μg; 2-methyl-1,4-naphthoquinone, 25 μg; and vitamin B_{12}, 1 μg. Food and water were given *ad lib.* except during the collection of urine when food was withheld. Twenty-four-hour urine samples were collected under toluene by placing individual animals in small wire cages over glass funnels designed to minimize the mixing of urine and feces. Urine was not collected more often than at 2-day intervals. At least 3 urine collections were obtained from each animal, and creatine and creatinine were determined in appropriate aliquots of the urine by minor modifications of the Folin method (7).

After the animals had received the purified diet for approximately 1 month, each of 6 dystrophic mice and 6 of their normal litter mates was given an intraperitoneal injection of 1 μc (0.5 μc per μmole) of creatine-1-C^{14} per 25 g of body weight. Fifteen and 30 minutes after injection, 0.01-ml samples of blood were taken from a tail vein for C^{14} assay. Four animals from each group were killed 30 minutes after the injection, and 2 animals from each group were killed 38 days after the injection. One normal mouse (no. 6) was given an intraperitoneal injection of 1 μc of the creatine-1-C^{14} when it weighed 30 g, and it was not killed until 47 days after the injection. The concentration and specific activity of skeletal muscle creatine were determined by slight modifications of previously described methods (8, 9). Similar methods were used to measure heart creatine concentration and specific activity, but it was usually necessary to pool two hearts to obtain sufficient tissue for analysis. The major portion of the radioactive creatine was assumed to be intracellular. No corrections were made for the small amount of labeled creatine that may have been extracellular.

At intervals of 2 or more days, individual 24-hour urine collections were obtained from the mice that were kept 38 days or longer following creatine-1-C^{14} injection. Creatinine was separated from creatine by adjusting the 24-hour urine collection to pH 4 and mixing with Lloyd's reagent to adsorb creatinine. After washing the Lloyd's reagent three times with 5-ml portions of 0.01 N HCl,

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[1] The definition of this and other related terms may be found in the paper by Zilversmit (5).

[2] Vitamin-free casein purchased from Nutritional Biochemicals Corp., Cleveland, Ohio.

the creatinine was removed by washing twice with 3-ml portions of 0.5 N NaOH. The sodium hydroxide washings were acidified, and the specific activity of the creatinine was obtained by measuring its concentration and isolating creatinine zinc chloride after addition of carrier creatinine (9). This method eliminated over 99 per cent of creatine-1-C^{14} added to urine samples containing unlabeled creatine and creatinine.

In order to obtain adequate urine for the determination of the specific activity of both creatine and creatinine it was necessary to pool three 24-hour urine collections from each mouse. Three urine collections, obtained during the last week before the animals were killed, were pooled and aliquots were taken for creatine and creatinine determination and for isolation of creatinine. Another aliquot of the pooled urine samples was made approximately 1 N by adding concentrated HCl and heated in an autoclave at 118° C for 45 minutes. This procedure converted creatine to creatinine. The specific activity of the creatinine isolated from the autoclaved urine represented the average of the creatinine and creatine. Since the concentrations of creatine and creatinine and the specific activity of creatinine had been determined for the pooled urine samples, it was possible to calculate the specific activity of creatine.

All creatinine zinc chloride samples were counted in metal planchets with an ultra-thin window continuous gas-flow Geiger tube, and the observed counts were corrected to infinite thinness. The blood samples were mixed with 1 ml of a 1:1 mixture of acetone and water, dried in metal planchets and counted with an end-window Geiger tube. Therefore, the actual counts obtained with blood samples and creatinine zinc chloride samples are not comparable.

RESULTS

Both groups of animals continued to gain weight slowly on the purified diet, as is shown in Table I. The dystrophic mice weighed only about 65 per cent as much as their normal litter mates but, since all the animals gained weight throughout the experiment, it is apparent that involvement of the jaw muscles by the myopathy did not induce severe starvation.

TABLE I

Weight changes in normal and dystrophic mice

Age	Weight	
	Normal	Dystrophic
days	*g*	
67	21.0	15.0
77	23.7	15.4
85	24.6	15.3
97	24.9	15.9
104	25.3	16.4
135*	26.6	17.1

* Only 2 animals remained in each group at this time. The other weights are the averages of 4 to 6 animals.

The combined urinary excretion of creatine and creatinine (Table II) was similar in the two groups, although the dystrophic mice weighed less. The amount of creatinine excreted per gram of body weight was also similar in the two groups of animals, but the urinary creatine to creatinine ratio was higher in the dystrophic mice. The concentration of creatine was reduced in the skeletal muscle but not in cardiac muscle of the dystrophic mice. These observations agree in general with the report of Kandutsch and Russell (11) but differ somewhat with the findings of Perkoff and Tyler (12) who reported a very low urinary excretion of creatinine by the dystrophic mouse.

The specific activities of creatine of some of the tissues following creatine-1-C^{14} injection are given in Table III. The radioactivity present in the blood at 15 and 30 minutes following injection indicates that both groups of animals received similar doses of creatine-1-C^{14} and that the peak of

TABLE II

Effects of muscular dystrophy on creatine storage and excretion in mice

Measurement	Normal	Dystrophic	p*
Urinary creatine† + creatinine (μg/day)	599 [6]‡	649 [5]	>0.5
Urinary creatinine (μg/day/g body wt)	13.0[6]	14.1 [5]	>0.2
Urinary creatine/creatinine	1.15 [6]	2.26 [5]	<0.01
Skeletal muscle creatine (mg/g)	3.25 [7]	2.14 [6]	<0.001
Heart creatine (mg/g)	1.38 [7]	1.20 [6]	>.04

* Probability that the differences observed between normal and dystrophic mice are due to chance (10).
† Creatine concentrations are expressed as creatinine.
‡ Numbers in brackets indicate the number of animals used.

TABLE III

Incorporation of creatine-1-C^{14} into various tissues of the mouse

Measurement	Time after injection of creatine-C^{14}	Normal	Dystrophic	p*
Skeletal muscle creatine (cpm/μmole)	30 min	351 [4]	635 [4]	<0.001
Skeletal muscle creatine (cpm/μmole)	38 days	508 [2]	266 [2]	<0.05
Heart creatine (cpm/μmole)	30 min	2,710 [4]	3,380 [4]	>0.40
Heart creatine (cpm/μmole)	38 days	191 [2]	97 [2]	
Blood creatine (cpm/ml blood)	15 min	2,075 [4]	1,875 [4]	>0.50
Blood creatine (cpm/ml blood)	30 min	1,600 [4]	1,375 [4]	>0.50

* See footnotes (*‡) to Table II.

radioactivity in the blood had been reached earlier than 30 minutes after injection. The specific activity of skeletal muscle creatine in the dystrophic mice was higher than normal 30 minutes after injection and lower than normal 38 days after injection. This indicates that the turnover time of muscle creatine is shorter in the dystrophic mouse. In both groups of animals the specific activity of heart creatine 30 minutes after injection was about seven times that of skeletal muscle. The reduced turnover time of heart creatine was confirmed by finding specific activities lower than that of skeletal muscle 38 days after injection of labeled creatine.

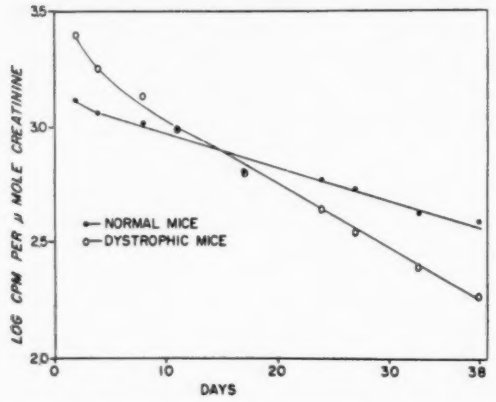

FIG. 1. RADIOACTIVITY OF URINARY CREATININE. Creatinine was separated from creatine by using Lloyd's reagent. The creatinine was then isolated for counting as creatinine zinc chloride by a carrier procedure. There were two female mice in each group.

The logarithm of the specific activity of urinary creatinine is plotted against time in Figure 1. It is apparent that the specific activity of the urinary creatinine is diminishing much more rapidly in the dystrophic mice than in their normal litter mates. In both groups of animals the curve is not a straight line until about 10 days following the injection of labeled creatine. If the straight portion of the curve is used to estimate the half-life of body creatine, half-lives of 20 and 11 days are obtained for the normal and dystrophic mice, respectively.

In Table IV the specific activity of creatinine in the urine collected on the day the animals were killed is compared with the specific activity of the

TABLE IV

Muscle creatine and urinary creatinine specific activities *

Animal	Condition	Muscle creatine	Urinary creatinine†
2‡	Normal	546	377
5	Normal	469	409
6§	Normal	497	314
2	Dystrophic	241	174
5	Dystrophic	290	195

* Counts per minute per μmole of creatine.
† The urine was collected during the last 24 hours before the animals were killed.
‡ Animals having the same number are litter mates.
§ This mouse was killed 47 days following injection of creatine-C^{14}; the other animals were killed 38 days after injection.

TABLE V

Urinary creatine and creatinine specific activity *

Animal	Condition	Creatine	Creatinine
2	Normal	296	415
5	Normal	355	421
6	Normal	238	378
2	Dystrophic	95	249
5	Dystrophic	122	252

* Counts per minute per μmole of creatine. Data were obtained from urine collected during the week before the mice were killed. No. 6 was killed 47 days after injection of creatine-C^{14}. The other animals were killed 38 days after injection.

skeletal muscle creatine from the same animal. The urinary creatinine in every case has a considerably lower specific activity than has the skeletal muscle creatine. It is not likely that this is due to the transformation of relatively large quantities of unlabeled creatine to creatinine while the urine was being collected, since the urinary creatine had an appreciable specific activity (Table V) a short time before the animals were killed.

DISCUSSION

Since most of the creatine of the body is located within skeletal muscle and since there is no evidence for enzymatic formation of creatinine (13, 14), it is commonly assumed that urinary creatinine has its origin largely in skeletal muscle. However, some of the data from the present experiment indicate that this is not a valid assumption for the mouse. When the logarithm of urinary creatinine specific activity is plotted against time, a straight line is not obtained until about 10 days following the injection of labeled creatine. This suggests that during the earlier time intervals there are two or more sources of radioactive urinary creatinine. If the curved portions of the graphs in Figure 1 are due to the formation of creatinine in sites other than skeletal muscle, then these sources of creatinine have a relatively short turnover time. It would, therefore, be expected that urinary creatinine, isolated a short time after administering labeled creatine, would contain a higher concentration of the label than would total body creatine (largely skeletal muscle creatine). This phenomenon has been observed in rats (15).

Further evidence against the unique origin of creatinine from skeletal muscle creatine is provided by the finding of a low urinary creatinine specific activity in comparison with that of skeletal muscle creatine (Table IV). The percentage of urinary creatinine that has its origin in skeletal muscle can be estimated by dividing urinary creatinine specific activity by skeletal muscle creatine specific activity and multiplying by 100. The calculation is based on the assumption that skeletal muscle creatine is the only body pool of creatine that is highly labeled 38 days following a single injection of creatine-1-C^{14}. The results of this calculation are recorded in Table VI. If the creatine outside the skeletal muscle were still significantly labeled at the time these specimens were obtained, then the numbers shown in the table would be too large. Thus it is evident that 25 per cent or more of the urinary creatinine of these mice did not come from skeletal muscle. This large source of creatinine might account for the excretion of normal amounts of urinary creatinine (based on body weight) by the dystrophic mice even though they exhibited low concentrations of creatine in skeletal muscle (Table II).

The assumption that only skeletal muscle creatine is highly labeled 1 month after a single injection of creatine-1-C^{14}, permits the estimation of the percentage of urinary creatine which is derived from skeletal muscle creatine. The urinary creatine specific activity divided by the muscle creatine specific activity and multiplied by 100 would give the percentage of the urinary creatine that came from skeletal muscle. Table VI records the results of this calculation. The values obtained are slightly high because the urine collections were obtained a few days before the animals were killed. Nevertheless, it is evident that only about 50 per cent of the urinary creatine represents

TABLE VI

Contribution of skeletal muscle to urinary creatine and creatinine

Animal	Condition	Urinary creatinine derived from muscle creatine*	Urinary creatine derived from muscle creatine*
		%	%
2	Normal	69.0	54.2
5	Normal	87.0	75.7
6	Normal	63.2	47.9
2	Dystrophic	72.2	39.4
5	Dystrophic	67.2	45.4

* See text for method of calculation.

TABLE VII

Daily loss of skeletal muscle creatine as urinary creatine and creatinine

Animal	Condition	Lost as urinary creatine*	Lost as urinary creatinine*
		%	%
2	Normal	0.43	0.51
5	Normal	0.73	1.14
6	Normal	0.53	0.85
2	Dystrophic	1.67	2.00
5	Dystrophic	2.44	2.05

* See text for method of calculation.

newly synthesized creatine and that the relative amount of newly synthesized creatine which is excreted in the urine is similar in the two groups of animals.

The percentage of skeletal muscle creatine excreted daily as urinary creatine or creatinine can be estimated by dividing the amount of urinary creatine or creatinine that has its origin in skeletal muscle (based on the percentages previously calculated) by the estimated amount of skeletal muscle creatine and multiplying by 100. The amount of skeletal muscle was estimated to be 40 per cent of the total body weight, and this was multiplied by the concentration of skeletal muscle creatine for a particular animal in order to arrive at the total amount of skeletal muscle creatine for that animal. The errors of this estimation would tend to minimize the differences between the dystrophic and normal mice. Even so, it is apparent from Table VII that the relative daily loss of skeletal muscle creatine as urinary creatine or creatinine is twice as great in the dystrophic mice as in their normal litter mates. This reflects the greatly reduced turnover time of skeletal muscle creatine in the dystrophic mice.

A reduced turnover time of skeletal muscle creatine in the dystrophic mice means that the molecules of creatine reside for a shorter than normal period of time in the muscle. There are two possible explanations for this finding; either there is defective metabolism of creatine by skeletal muscle, or the creatine within the muscle must be in rapid equilibrium with a relatively larger extramuscular pool of creatine. Which of these explanations is chosen depends in part upon the nature of the mechanisms that maintain muscular creatine levels.

The mechanisms responsible for maintaining the high muscle creatine concentrations are not known, but active transport of creatine across the cell membrane and intracellular trapping of creatine are two possible explanations. If the latter possibility were the primary mechanism, a rapid exchange of creatine across cell membranes would not be expected. On the other hand, if an active transport system maintained the high concentrations of creatine within the cell it would be possible for labeled intracellular creatine to exchange rapidly with nonlabeled extracellular creatine. If a rapid exchange normally occurs, the difference in pool size between normal and dystrophic animals might be enough to account for the differences in turnover times of skeletal muscle creatine. However, this would not explain the low concentration of creatine in the skeletal muscle of the dystrophic mice, which is present regardless of whether the concentration is based on wet weight of tissue, fat-free dry weight of tissue (11), or noncollagen nitrogen (16).

A decrease in the intracellular concentration of creatine must be the result of a higher rate of loss of creatine than its rate of entry. If the rate of entry of creatine into skeletal muscle is normal or increased and the rate of loss is abnormally high, it would suggest that a mechanism which normally prevents the loss of creatine from the cell is impaired by the dystrophic process. If the rate of entry of creatine into the skeletal muscle is lower than normal and its rate of loss normal or decreased, it would suggest that a mechanism for the transport of creatine into the cell is not functioning properly. If the rate of entry of creatine into the muscle were low and the rate of exit high, it would suggest that both the above mechanisms for maintaining muscle creatine concentrations are operative but functioning abnormally.

The uptake of creatine-1-C^{14}, relative to the amount of creatine in skeletal muscle, is increased in the dystrophic mice (Table III), and it may be calculated that the total amount of creatine-1-C^{14} that enters each gram of skeletal muscle is similar in the two groups of animals. Coupling this observation with the reduced turnover time of skeletal muscle creatine and the low concentration of creatine in the muscle of the dystrophic mice leaves little doubt that there is a defect in a mechanism which normally prevents the loss of creatine from the cell. The excess loss of creatine could result

from increased conversion of creatine to creatinine, from leakage of creatine out of the muscle, or from a combination of both possibilities. Because of the limitations of the present experiment it is not possible to exactly define the defect, but it is clear that the metabolism of creatine by skeletal muscle is abnormal in mice with hereditary muscular dystrophy. The possibility thus arises that the muscular weakness or wasting of these mice may be related to the abnormal metabolism of creatine.

A similar defect in the muscular metabolism of creatine occurs in vitamin E-deficient animals (9, 17) which also exhibit muscular weakness and wasting, but the primary change in creatine metabolism in progressive muscular dystrophy in man is thought to be a decreased total uptake of creatine because of the smaller muscle mass (18, 19). Since the latter might be interpreted as indicating normal metabolism of creatine by the surviving skeletal muscle, it should be pointed out that some of the observations in patients with progressive muscular dystrophy suggest that the changes in creatine metabolism are similar to the changes found in dystrophic mice.

The experiments with humans which are most comparable with those reported here were performed by Roche (18) and Benedict (19) and their co-workers. These investigators administered N^{15}-labeled glycine to normal subjects and to patients with progressive muscular dystrophy. Urinary creatinine was subsequently isolated from both groups and urinary creatine was isolated from the dystrophic patients. Two of the findings are similar to those reported here. The N^{15} concentration of the urinary creatinine was always higher in the dystrophic than in the normal subjects, and in all of the dystrophic patients the N^{15} content of urinary creatinine decreased during the 2-week observation period. By contrast, the N^{15} concentration of urinary creatinine from the normal subjects did not change during the observation period. Because of these findings it was suggested that the fraction of muscle creatine converted to creatinine was increased in progressive muscular dystrophy (19).

It is well established for the rat that urinary creatinine is derived from body creatine (20). If this is also true for man, a decrease in the N^{15} concentration of urinary creatinine would indicate a dilution of body creatine with unlabeled creatine. However, Roche and Benedict and their associates (18, 19) found that the N^{15} concentration of urinary creatine was much higher than that of urinary creatinine even when the N^{15} concentration of urinary creatinine was decreasing. These two observations appear to be contradictory, unless it is assumed that the source of urinary creatine is different from the source maintaining tissue concentrations. Based on current knowledge of creatine metabolism, such a circumstance appears highly unlikely. Since glycine enters into so many metabolic pathways, more easily interpretable data might be obtained by using labeled creatine to investigate the metabolism of creatine by skeletal muscle in progressive muscular dystrophy. Until other evidence is available, it should not be assumed that the muscular metabolism of creatine is normal in patients with progressive muscular dystrophy.

SUMMARY

The urinary excretion of creatine and creatinine by mice with hereditary muscular dystrophy and by their normal litter mates was measured while the animals were receiving a purified diet. Normal and dystrophic mice were then given a single intraperitoneal injection of creatine-1-C^{14} and killed either 30 minutes or several days after the injection. The concentration and specific activity of the creatine of heart and skeletal muscle were measured in all the animals, and urinary creatine and creatinine specific activities were determined for the animals that were allowed to live for 38 days or longer after the injection of labeled creatine.

The combined excretion of urinary creatine and creatinine was similar in the two groups of animals, but the ratio of urinary creatine to creatinine was increased in the dystrophic mice. The urinary excretion of creatinine expressed as a function of body weight was not decreased in the dystrophic mice. The concentration of creatine was reduced in skeletal muscle but not in cardiac muscle of the dystrophic mice.

The turnover time of both the total body creatine and skeletal muscle creatine was reduced in the dystrophic mice. These findings indicate that the low concentration of creatine in the skeletal muscle of the dystrophic mice results from an impaired ability to retain creatine.

The turnover time of heart creatine was considerably less than that of skeletal muscle in both groups of animals, but no differences between the two groups were found.

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THE EFFECTS OF INFUSIONS OF CHLOROTHIAZIDE ON URINARY DILUTION AND CONCENTRATION IN THE DOG

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It has been observed that the saluretic agent chlorothiazide (6-chloro-7-sulfamyl-1,2,4-benzothiadiazine-1,1 dioxide) and its analogs produce alterations in the process of urinary dilution which differ from those expected from a simple increase in electrolyte excretion (1–3). Heinemann, Demartini and Laragh (2) observed in human subjects undergoing water diuresis that the augmented electrolyte excretion resulting from the injection of chlorothiazide was associated with no increase, or a fall, in the clearance of free water. In a single experiment the authors observed no effect of the drug on the elaboration of a concentrated urine, and suggested that chlorothiazide may in part interfere with the reabsorption of electrolyte at sites in the distal convoluted tubule where urinary dilution normally occurs. The absence of an effect on the concentrating mechanism has been confirmed by others (4).

Crawford and Kennedy (3) found that the continuous administration of chlorothiazide (or hydrochlorothiazide) to patients with pituitary or nephrogenic diabetes insipidus, and to rats with pituitary diabetes insipidus, resulted in decreased daily volumes of urine with increased concentrations of solute. Similar effects of this agent and its analogs have been reported by numerous other investigators (5–10).

The following studies were undertaken to investigate further the immediate effects of chlorothiazide on the processes of urinary concentration and dilution in the dog. The changes observed during water diuresis were similar to those observed by others in man. A definite effect of this drug during the elaboration of concentrated urine was also observed, and has been interpreted as supporting the previous suggestion that this agent may interefere with electrolyte reabsorption at sites in the nephron where urinary dilution occurs.

PROCEDURE

Water diuresis. Eight experiments were performed on 2 trained unanesthetized mongrel female dogs. The dogs were given their usual diet and fluid *ad lib.* prior to the experiment. At the onset of the experiment, a water load equivalent to 5 per cent of the body weight was administered through a gastric tube. Inulin in 2.5 per cent dextrose in water was infused at a constant rate of 10 to 14 ml per minute. Urine was collected by free flow through an indwelling catheter with the dog in the upright position. After at least 1 hour had been allowed for equilibration, urine specimens were collected and the rate of flow and osmolality determined. When these were stable for 3 consecutive 10-minute periods, collection of "control" periods was begun. Venous blood samples were collected every 30 to 40 minutes by free flow through an indwelling needle into a heparinized tube.

In 4 experiments, after the collection of 3 "control" periods, chlorothiazide (50 mg per kg body weight) was administered intravenously and a like amount was given by constant infusion each hour. Four 10-minute periods were then collected. The acute change in solute excretion and free water clearance produced by the drug under these conditions was thus determined.

In order to examine the relationship between C_{osm} and C_{H_2O} during water diuresis, 2.5 per cent mannitol in water was infused in 3 experiments to produce progressively increasing rates of solute excretion. The relationship was re-examined during the administration of chlorothiazide. The drug was administered either before the 2.5 per cent mannitol infusion was begun or after the mannitol had been infused and a high rate of solute excretion attained. In the former instance the mannitol infusion was then started in order to increase solute excretion, and in the later instance it was then stopped in order to decrease solute excretion. The effect of chlorothiazide was thus examined under conditions of both increasing and decreasing rates of solute excretion.

The effect of an equivalent dose of acetazolamide was examined in the same manner.

Maximal hydropenia and antidiuretic hormone. In order to examine the effects of chlorothiazide on urinary concentration, 11 experiments were performed on 5 trained unanesthetized mongrel female dogs. The dogs were

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dehydrated for 48 hours and 5 U of vasopressin (Pitressin Tannate in Oil) was administered intramuscularly the evening preceding the experiment.

A continuous infusion of inulin in isotonic saline was maintained at a constant rate throughout the experiment. Aqueous vasopressin was added to the solution, the pH having been adjusted to 5.5 with acetic acid, and infused at the rate of 50 mU per kg of body weight per hour during the course of the experiment. After a period of equilibration, urine and blood specimens were collected as indicated above. The periods varied from 5 to 20 minutes depending upon the rate of urine flow.

In order that the relationship between C_{osm} and $T^c_{H_2O}$ be examined over a wide range of solute excretion, one of the following hypertonic solutions was infused: *1)* 7 per cent mannitol in water containing 50 mmoles NaCl per L; *2)* 5 per cent mannitol in water containing 50 mmoles NaCl per L; *3)* 4 per cent mannitol in water containing 150 mmoles NaCl per L; *4)* 2.5 per cent mannitol in water containing 150 mmoles NaCl per L; *5)* 200 mmoles NaCl per L. The stability of the relationship was determined by discontinuing the infusion after a high rate of solute excretion had been obtained and re-examining the C_{osm}-$T^c_{H_2O}$ relationship as solute excretion was decreasing.

Chlorothiazide, in the same dosage as noted above, was administered when solute excretion was either at a high rate (after *one* C_{osm}-$T^c_{H_2O}$ curve had been obtained with the hypertonic solution) or after solute excretion had returned to a low rate (after *two* C_{osm}-$T^c_{H_2O}$ curves had been obtained with the hypertonic solution). In either case, adjusting the rate of infusion of the hypertonic solution permitted observations through a wide range of solute excretion in the presence of chlorothiazide. In all experiments one or more C_{osm}-$T^c_{H_2O}$ curves were obtained with hypertonic mannitol and/or saline, and one curve was obtained during the administration of chlorothiazide.

Chemical methods. Inulin was measured in plasma and urine by the method of Walser, Davidson and Orloff (11). Sodium and potassium were determined by flame photometry. Osmolality was determined cryoscopically by using the freezing point depression apparatus of Bowman, Trantham and Caulfield (12).

RESULTS

Water diuresis. Prior to the administration of chlorothiazide, the relationship between C_{osm} and C_{H_2O} was similar in all experiments. The osmolal clearance prior to the infusion of the hypotonic solution of mannitol ranged from 0.8 to 1.9 ml per minute and the C_{H_2O} ranged from 10.9 to 13.1 ml per minute. When mannitol was infused, the osmolal clearance slowly increased to a maximum of 6.5 ml per minute and the free water clearance increased progressively to a maximum of 15.1 ml per minute (Figure 1, control). The free water

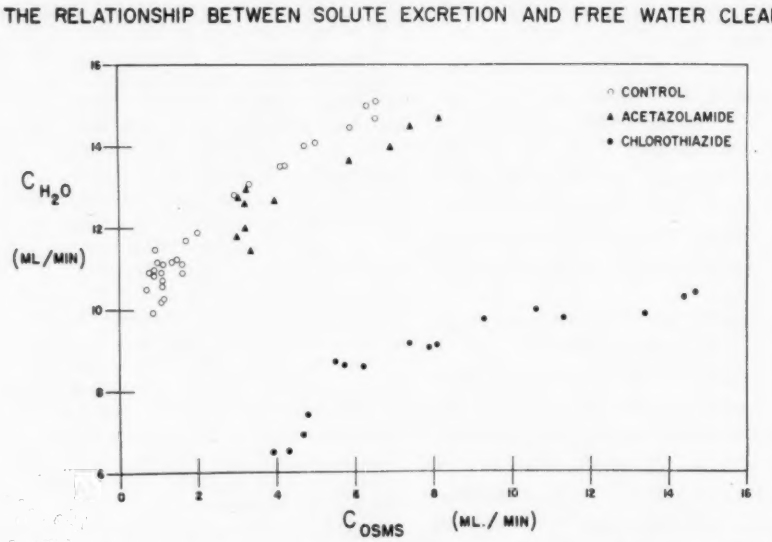

FIG. 1. COMPOSITE OF 5 STUDIES DURING WATER DIURESIS IN A SINGLE DOG. Control points demonstrate increasing clearance of free water with increasing osmolal (mannitol) clearance. A similar relationship is shown during the administration of acetazolamide. Chlorothiazide points represent 3 different experiments; control points immediately preceding each of these experiments are grouped at the left of the upper curve.

TABLE I

The effects of chlorothiazide on the excretion of water and electrolyte during water diuresis

Experiment	Time	V	U_{osm}	C_{osm}	$U_{Na}V$	U_KV	C_{H_2O}	C_{In}	per 100 ml GFR V	per 100 ml GFR C_{osm}	per 100 ml GFR C_{H_2O}
	min	*ml/min*	*mOsm/kg*	*ml/min*	*μEq/min*	*μEq/min*	*ml/min*	*ml/min*	*ml/min*	*ml/min*	*ml/min*
A5		900 ml H_2O by gastric tube, 2.5% dextrose in water, 12.0 ml/min i.v.									
	0–10	14.2	30	1.6	11	8	12.6	109	13.0	1.5	11.6
	10–20	14.7	32	1.8	12	9	12.9	109	13.5	1.6	11.9
	20–30	14.6	32	1.8	12	8	12.8	111	13.2	1.6	11.6
		Chlorothiazide, 5 mg/kg and 5 mg/kg/hr i.v.									
	30–40	15.4	85	5.0	418	86	10.5	94	16.5	5.3	11.2
	40–50	14.4	109	6.0	558	98	8.5	89	16.3	6.7	9.6
	50–60	13.6	109	5.7	544	94	7.9	88	15.5	6.5	9.0
	60–70	12.7	110	5.2	485	89	7.4	87	14.7	6.1	8.6
J9		1,000 ml H_2O by gastric tube, 2.5% dextrose in water, 12.0 ml/min i.v.									
	0–10	12.0	22	1.0	7	7	10.9	109	11.0	0.9	10.0
	10–20	12.0	24	1.1	7	7	10.9	110	10.9	1.0	9.9
	20–30	12.2	21	1.0	8	7	11.2	117	10.4	0.9	9.6
		Chlorothiazide, 5 mg/kg and 5 mg/kg/hr i.v.									
	30–40	14.8	92	5.2	513	95	9.6	101	14.7	5.2	9.5
	40–50	14.4	110	6.1	638	119	8.3	107	13.5	5.7	7.8
	50–60	14.4	104	5.7	596	109	8.6	113	12.7	5.1	7.6
	60–70	14.2	101	5.5	567	102	8.7	119	11.9	4.6	7.3

clearance during the infusion of chlorothiazide was consistently lower than that noted with mannitol alone at all rates of solute excretion examined (Figure 1).

In four experiments, chlorothiazide was administered before mannitol was infused. The protocols of two of these experiments are shown in Table I. There was a mean increase in osmolal clearance of 4.2 to 5.4 and a mean decrease in free water clearance of 2.4 to 3.2 ml per minute per 100 ml glomerular filtration rate (GFR). When the free water clearance obtained during the administration of chlorothiazide was compared with the free water clearance obtained with mannitol alone *at the same rate of solute excretion,* there was a net decrease in free water clearance of 5.3 to 5.8 ml per minute per 100 ml GFR. It is apparent from Table II that in all four experiments the increased osmolal clearance closely approximated the decreased free water clearance (mean $C_{osm}/C_{H_2O} = 0.87$).

In the four experiments noted above there was an initial increase in the rate of urine flow following the administration of chlorothiazide. In three of these there was a subsequent decrease in the rate of flow during the 40 minutes of drug ad-

TABLE II

The relationship between changes in the rates of osmolal and free water clearance produced by chlorothiazide during water diuresis

Experiment	Mean C_{osm} Before chloro.	Mean C_{osm} After chloro.	ΔC_{osm} (increase)	Mean C_{H_2O} After chloro.	Mean C_{H_2O} During mannitol*	ΔC_{H_2O} (decrease)	$\frac{\Delta C_{osm}}{\Delta C_{H_2O}}$
	ml/min		*ml/min*	*ml/min*		*ml/min*	
J1	0.9	5.3	4.4	8.2	13.5	5.3	0.83
J2	0.9	5.1	4.2	7.6	13.3	5.7	0.74
A1	1.4	6.5	5.1	9.1	14.8	5.7	0.89
A2	1.4	6.8	5.4	9.2	15.0	5.8	0.93

* C_{H_2O} during water diuresis with superimposed mannitol infusion selected at rates of osmolal clearance (C_{osm}) equal to rates obtained during the respective experiments with chlorothiazide.

ministration. This decreased flow rate was associated with a persistent fall in glomerular filtration rate which averaged 13, 20 and 22 per cent, respectively. The mean rate of flow was *increased* 1.5, 2.3 and 2.8 ml per minute per 100 ml GFR above prechlorothiazide levels when the adjustment for filtration rate was made. The protocol of one of these experiments is shown in Table I, A5. The one experiment in which the rate of urine flow did not fall is shown for comparison (Table I, J9). In this latter experiment the fall in filtration rate following the administration of chlorothiazide was only transient. The increased rate of solute excretion and decreased free water clearance are similar in both experiments in spite of these differences in filtration rate.

In an experiment in which acetazolamide was administered, the mean osmolal clearance increased 2.4 ml per minute, and the filtration rate fell 14 per cent, values similar to those noted above following chlorothiazide administration. As can be seen in Figure 1, the free water clearance increased during the administration of acetazolamide in a manner similar to that with mannitol alone. Adjustment for changes in filtration rate did not alter this identity.

Maximal hydropenia and antidiuretic hormone. A consistent relationship between C_{osm} and $T^c_{H_2O}$ was noted when hypertonic mannitol and/or saline was infused. If the rate of solute excretion was initially low, a progressive rise in $T^c_{H_2O}$ with increasing C_{osm} was generally observed. With further increases in the rate of solute excretion, a "plateau" was apparent with only minimal changes in $T^c_{H_2O}$ (Figure 2, control). In six experiments, the plateau was followed by a progressively decreasing $T^c_{H_2O}$ as the rate of solute excretion was further increased (Figure 3, control). In two of the latter experiments an initial rise in $T^c_{H_2O}$ was not observed, and the urine became hypotonic to plasma, resulting in the excretion of free water at high rates of solute excretion

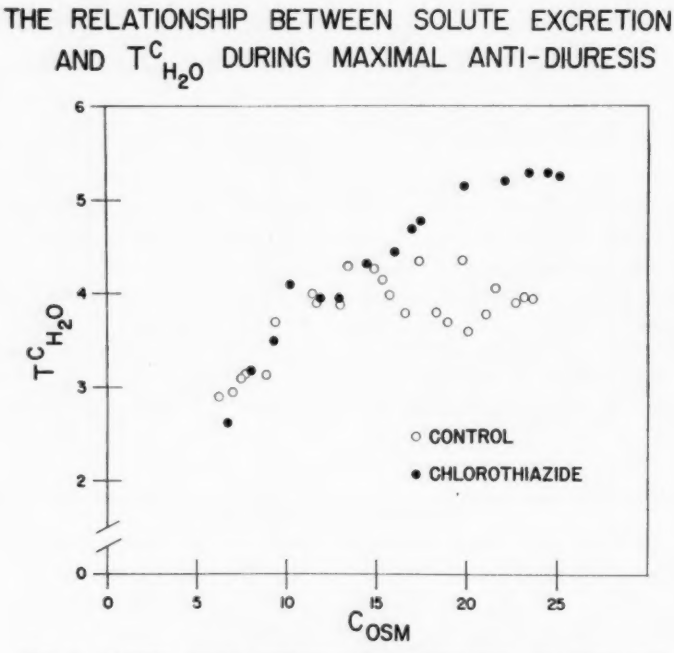

FIG. 2. CONTROL POINTS REPRESENT BOTH INCREASING AND DECREASING RATES OF SOLUTE EXCRETION. Chlorothiazide was administered when solute excretion had returned to a low level (see text). In the presence of the drug the curve did not differ from control at low rates of solute excretion. At higher rates of solute excretion, $T^c_{H_2O}$ during the administration of chlorothiazide is distinctly greater than without the agent.

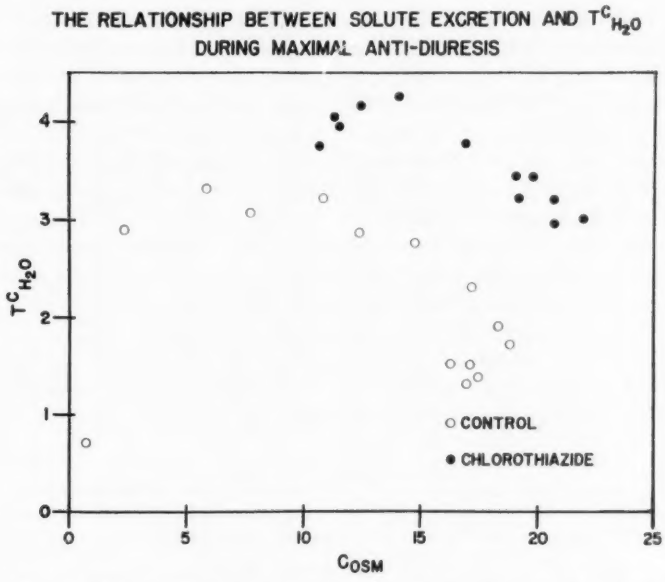

FIG. 3. THE CONTROL POINTS REPRESENT AN EXPERIMENT IN WHICH $T^{c}_{H_2O}$ PROGRESSIVELY DECREASED AT HIGH RATES OF SOLUTE EXCRETION. Chlorothiazide was administered while high rates of solute excretion were maintained, and $T^{c}_{H_2O}$ immediately increased. Solute excretion was then allowed to decrease and $T^{c}_{H_2O}$ remained greater than control points.

(Table III). These relationships were qualitatively the same regardless of which hypertonic solution was infused.

In each experiment, the C_{osm}-$T^{c}_{H_2O}$ relationship during the infusion of hypertonic solution was reproducible. This is well illustrated in those experiments in which two curves were obtained with the hypertonic solution during a 3 to 5 hour period by first increasing and then decreasing the rate of infusion. The "control" curve shown in Figure 2 is a composite plot of all of the periods from *two* curves obtained in this way. The minimal scatter emphasizes the stability of the C_{osm}-$T^{c}_{H_2O}$ relationship. This is also apparent in the protocol of Table III. (For example, during progressively *increasing* solute excretion, at a C_{osm} of 9.7 ml per minute the $T^{c}_{H_2O}$ is 0.6 ml per minute. During progressively *decreasing* solute excretion, at a C_{osm} of 10.4 ml per minute, the $T^{c}_{H_2O}$ is 0.5 ml per minute.) Differences, when noted, are both quantitatively and qualitatively quite distinct from those obtained during the administration of chlorothiazide.

In spite of this stability during each experiment, there was often marked variability in the C_{osm}-$T^{c}_{H_2O}$ relationship between experiments on different dogs and between experiments on the same dog on different days. It was therefore necessary to obtain at least one C_{osm}-$T^{c}_{H_2O}$ curve during the infusion of hypertonic mannitol-saline solution and one curve during the administration of chlorothiazide in each experiment in order to make valid comparisons.

Within 5 to 15 minutes after chlorothiazide was administered, the osmolal clearance increased 2.1 to 4.9 ml per minute (mean 3.8) and the rate of urine flow increased 1.2 to 4.5 ml per minute (mean 3.0). Following the administration of chlorothiazide, in three experiments the filtration rate fell less than 5 per cent. In eight experiments the decrease was 7 to 15 per cent during the first 20 minutes but in seven of these the filtration rate returned to control levels or above in spite of the continued chlorothiazide infusion. The characteristic C_{osm}-$T^{c}_{H_2O}$ relationship noted during the administration of the drug was neither dependent upon nor qualitatively altered by these variations in filtration rate.

The C_{osm}-$T^c_{H_2O}$ relationship during the administration of chlorothiazide had a characteristic pattern when compared with the relationship obtained during the infusion of the hypertonic solutions alone. This pattern was noted in experiments in which the drug was initially administered at either high rates of solute excretion (Figure 3) or at low rates of solute excretion (Figure 2, Table III). At low rates of solute excretion (corresponding to the phase of rapidly rising $T^c_{H_2O}$ of the control curve), the $T^c_{H_2O}$ during chlorothiazide administration was not apparently different from the hypertonic mannitol-saline solutions alone at the same C_{osm} (Figure 2). At rates of solute excretion corresponding to the late part of the "plateau" of the "control" curve (Figure 2) and in all six experiments in which the rate of solute excretion during the infusion of the hypertonic solutions alone was great enough to obtain a falling $T^c_{H_2O}$ (Figure 2, Table III), the curves differed appreciably and consistently. The $T^c_{H_2O}$ obtained at these rates of solute excretion was greater during the administration of chlorothiazide than that obtained with the hypertonic solutions alone at equivalent rates of solute excretion. This divergence was noted at or slightly beyond the point of maximal $T^c_{H_2O}$ of the "control" curve. Once this divergence was apparent, the $T^c_{H_2O}$ with chlorothiazide remained greater than with the mannitol-saline solutions alone at the same

TABLE III

The effect of chlorothiazide on urinary concentration during maximal hydropenia and solute diuresis

Time	V	U_{osm}	C_{osm}	$T^c_{H_2O}$	C_{H_2O}	C_{In}	per 100 ml GFR V	per 100 ml GFR C_{osm}	per 100 ml GFR $T^c_{H_2O}$	per 100 ml GFR C_{H_2O}
min	*ml/min*	*mOsm/kg*	*ml/min*	*ml/min*	*ml/min*	*ml/min*	*ml/min*	*ml/min*	*ml/min*	*ml/min*
Inulin, 10 mg/min; vasopressin, 100 mU/kg/hr i.v.										
0–25	1.8	450	2.8	1.0						
Mannitol, 7% in 50 mmoles NaCl, 2.3 ml/min										
30–35	4.4	371	5.7	1.3		92	4.8	6.2	1.4	
35–40	5.5	347	6.6	1.1		95	5.7	6.9	1.2	
40–45	7.3	328	8.3	1.0		85	8.6	9.7	1.1	
Mannitol, 7% in 50 mmoles NaCl, 12.4 ml/min										
45–50	9.1	313	9.7	0.6		86	10.6	11.3	0.7	
55–60	14.0	297	14.0	0.0		83	16.9	16.9	0.0	
Mannitol, 7% in 50 mmoles NaCl, 22.0 ml/min										
65–70	18.2	291	17.6		0.6	79	23.1	22.4		0.7
75–80	23.3	286	22.3		1.0	79	29.5	28.2		1.3
Mannitol, 7% in 50 mmoles NaCl, discontinued										
85–90	23.4	285	22.4		1.4	76	31.2	29.4		1.8
95–100	15.5	296	15.2		0.3	78	19.8	19.5		0.3
100–105	12.2	298	12.0		0.2	77	15.8	15.5		0.3
105–110	9.9	318	10.4	0.5		82	12.1	12.8	0.8	
120–130	6.3	359	7.6	1.3		82	7.6	9.2	1.6	
140–155	3.9	419	5.5	1.6		94	4.1	5.8	1.7	
Chlorothiazide, 5 mg/kg and 5 mg/kg/hr i.v.										
165–170	4.4	431	6.3	1.9		95	4.7	6.7	2.0	
170–175	5.4	427	7.7	2.3		78	6.9	9.8	2.9	
Mannitol, 7% in 50 mmoles NaCl, 12.0 ml/min										
185–190	8.0	382	9.8	1.8		80	9.9	12.2	2.3	
195–200	10.8	368	12.9	2.1		78	14.0	16.6	2.6	
Mannitol, 7% in 50 mmoles NaCl, 22.0 ml/min										
205–210	14.0	353	16.1	2.1		78	18.1	20.8	2.7	
215–220	18.3	345	20.7	2.4		81	22.7	25.7	3.0	
225–230	21.0	337	23.3	2.3		79	26.7	29.6	2.9	
235–240	23.2	338	24.8	1.6		81	29.0	31.0	2.0	

rates of solute excretion. The maximal differences in $T^c_{H_2O}$ between the curves at the same rates of solute excretion were + 1.0 to + 3.5 ml per minute. The *maximal* $T^c_{H_2O}$ obtained during the administartion of chlorothiazide was often greater (1.0 to 1.8 ml per minute) than the *maximal* $T^c_{H_2O}$ obtained with the hypertonic solutions alone, as can be seen in Figures 2 and 3 and Table III.

DISCUSSION

The effects of chlorothiazide on the mechanisms of urinary dilution and concentration observed in the present studies may be interpreted in the light of current views as due to a single action of this agent on the renal tubule. The currently available evidence suggests that urinary dilution begins in the ascending limb of Henle's loop by a process which actively transports solute (sodium) from the lumen into the medullary interstitium without an equivalent movement of water (13, 14). Further dilution of the tubular fluid may occur in the distal convolution by the continued removal of solute, since in the absence of antidiuretic hormone (ADH) the movement of water out of this segment is restricted (13–15). The collecting duct, in the absence of ADH, is also relatively impermeable to water (13, 16), and the diluted fluid which enters this segment is unable to attain osmotic equilibrium with the surrounding medullary interstitium. It is likely that this relative impermeability to water of the distal convolution and collecting duct does not completely restrict the passive outward movement of water along a steep osmotic gradient. Thus, increasing rates of solute excretion during water diuresis may, by decreasing the transtubular osmotic gradient in the presence of increasing rates of flow within the lumen, result in further restriction of the outward flow of water from the distal convolution and collecting duct. As has been suggested, the increasing clearance of free water seen when increasing solute excretion is imposed on water diuresis may be explained in this manner (17, 18). The excretion of free water may also be increased by augmented reabsorption of sodium in the distal portions of the nephron. However, the relative importance of these two factors during solute diuresis cannot readily be assessed.

Unlike the results seen when mannitol was given during water diuresis, the administration of chlorothiazide uniformly resulted in a decreased clearance of free water. This is in agreement with the findings of others, and suggests that the action of chlorothiazide cannot be limited to diminishing the reabsorption of electrolyte in the proximal tubule. Further, if the increased osmolal clearance associated with this agent is envisioned as the result of interference with the reabsorption of electrolyte at a site where the tubular fluid is normally diluted, then a quantiative relationship should exist between the increased osmolal clearance and the decreased clearance of free water. Such a relationship was observed in the present studies. The decreases in C_{H_2O} closely approximated the increases in C_{osm} (when compared with values obtained during mannitol diuresis), suggesting that the major increase in electrolyte excretion following the administration of chlorothiazide resulted from interference with reabsorption at tubular sites where dilution occurs.

The observed effects of acetazolamide on the clearance of free water are in agreement with previous reports (19, 20), and the qualitatively opposite effects of the two drugs on this measurement make it unlikely that the effect of chlorothiazide on the diluting process is related to the inhibitory effect on carbonic anhydrase common to these two agents.

A similar tubular effect of chlorothiazide was detected in the animal elaborating a concentrated urine. In the absence of the drug, increasing rates of osmolal clearance during the infusion of vasopressin were associated with values for $T^c_{H_2O}$ which increased to a maximum and then either remained constant through the range of osmolal clearance examined, or progressively declined. In some instances dilute urine (C_{H_2O}) was excreted at high rates of osmolal clearance, despite the continued infusion of vasopressin. Such a fall in $T^c_{H_2O}$ at high rates of solute excretion has been occasionally observed by other investigators (18, 21, 22). Since it is this portion of the $T^c_{H_2O}$ curve that is affected by chlorothiazide, it is important to attempt an interpretation of the expected C_{osm}-$T^c_{H_2O}$ relationship in order that the action of the drug may be better defined.

The hypotonic tubular fluid produced in the ascending limb of Henle's loop is allowed to reach osmotic equilibrium with its surroundings in the

distal convolution through a membrane which is made highly permeable to water by the presence of ADH. The fluid will thus become isotonic with plasma in the latter portion of the distal convolution. In the collecting duct the passive movement of water is again enhanced by the presence of ADH, and the tubular fluid will approach osmotic equilibrium with the medullary interstitium which has been made hypertonic to plasma by the countercurrent systems of Henle's loop and the medullary capillaries. Under these conditions the calculated $T^c_{H_2O}$ would represent a true measure of the solute-free water abstracted from the collecting duct. However, as the rate of solute excretion is increased, the increased rate of flow through the distal nephron and the decreased transtubular osmotic gradient may result in a failure of complete passive osmotic equilibration of the diluted fluid which enters the distal convolution (18). Under these conditions the fluid entering the collecting duct would be hypotonic to plasma, and the calculated $T^c_{H_2O}$ would not represent a true measure of the solute-free water removed from the fluid within this segment. If the transport of solute into the medullary interstitium by the ascending limb of Henle's loop [and perhaps the collecting duct (23)] were proceeding at a maximal rate, then the delivery of hypotonic fluid to the collecting duct may impose a limit upon the maximal calculated $T^c_{H_2O}$ and result in its subsequent fall as solute excretion continues to increase. The externally calculated $T^c_{H_2O}$ would then represent the difference between the nonosmotically equilibrated water which enters the collecting duct and the solute free water abstracted by the medullary interstitium. This has been discussed in greater detail elsewhere (18). In support of this concept is the present observation that in some instances urine, hypotonic to plasma, was excreted at high rates of osmolal clearance, indicating that complete equilibration had not occurred even in the collecting duct. Although such marked falls in $T^c_{H_2O}$ were not observed in all studies, the qualitative similarity that exists throughout these C_{osm}-$T^c_{H_2O}$ curves suggests that the same basic mechanisms were operative.

Chlorothiazide, by interfering with the tubular reabsorption of solute without water at diluting sites, would result in less solute-free water within the distal convolution, and allow passive osmotic equilibration to occur more readily. Such an action might be expected to increase the maximal $T^c_{H_2O}$ and delay the subsequent fall which normally occurs. At lower rates of osmolal clearance, when $T^c_{H_2O}$ is progressively rising or is stable, passive osmotic equilibration in the distal convolution should be complete, and diminishing the dilution of the tubular fluid under these conditions should not affect the calculated $T^c_{H_2O}$ (provided the rate of solute transport into the medullary interstitium were not simultaneously affected). In the present studies chlorothiazide fulfilled these requirements. At rates of osmolal clearance associated with increasing values for $T^c_{H_2O}$ the administration of this agent did not alter these values. However, the presence of chlorothiazide resulted in maximal values for $T^c_{H_2O}$ greater than the maximal values obtained without the drug. Also, the administration of this agent uniformly resulted in increased values for $T^c_{H_2O}$ at rates of osmolal clearance which were associated with decreasing values in the absence of chlorothiazide. The failure of this drug to lower $T^c_{H_2O}$ at any level of solute excretion is consistent with the previous suggestion that its action is not manifest to any measurable degree in the ascending limb of Henle's loop, and that its primary site of action may be in the distal convolution (2). (However, since factors other than the rate of transport of sodium into the medullary interstitium may be important determinants of the measured $T^c_{H_2O}$, the exclusion of Henle's loop as a site of action of this drug may not be justified.) These conclusions are in contrast to those of Kessler, Hierholzer, Gurd and Pitts (24) who, by utilizing the "stop flow" method in dogs, concluded that the primary action of chlorothiazide on sodium reabsorption was within the proximal tubule. However, since all "stop flow" urine will ultimately be influenced by its passage through the more distal nephron, a quantitative discrimination of functions which occur both proximally and distally may be difficult.

These studies do not exclude the possibility that chlorothiazide, in addition to interfering with the reabsorption of electrolyte, enhances the passive movement of water across the epithelial membrane of the distal convolution and collecting duct in a fashion similar to that of ADH. If such were the case, the increases in $T^c_{H_2O}$ reported here

would necessitate that this agent increase the permeability to water of the distal portions of the nephron to an extent greater than that present during maximal ADH activity. In this study, as well as in those of others, chlorothiazide given during water diuresis never resulted in urinary concentrations as great as those that may be seen when vasopressin is given under similar circumstances (18, 25). The implication, thus, is that these two agents could increase the permeability of the tubular epithelium to water by separate and additive mechanisms. Further, an agent that increases the passive movement of water from the tubular urine should result in decreased urinary volumes with increased concentration of solute. Chlorothiazide uniformly produced an initial increase in urinary volume as the clearance of free water decreased. Since the increased osmolal clearance produced by the drug is quantitatively related to the decreased clearance of free water, it would seem convenient and justified to assign to this agent the single tubular action of interfering with solute reabsorption at sites of urinary dilution.

The administration of chlorothiazide was associated with variable changes in the rate of glomerular filtration, and it would appear unlikely that such changes as did occur were responsible for the observed effects on C_{H_2O} and $T^c_{H_2O}$. The constancy of the changes in C_{H_2O} produced by chlorothiazide, and the finding of similar changes in human subjects with nephrogenic diabetes insipidus (26), argue against the fact that the release of endogenous ADH produces any measurable effect on these results.

The mechanism whereby this drug results in an increase in urine osmolality acutely does not completely account for its chronic action in diabetes insipidus (see above). Additional factors are necessary to account for the associated fall in urine volume observed in these patients.

SUMMARY

Chlorothiazide was administered by continuous intravenous infusion to dogs undergoing maximal water diuresis. This agent resulted in an immediate fall in the clearance of free water (C_{H_2O}). This fall bore a consistent quantitative relationship to the increased osmolal clearance contributed by the drug.

Values for $T^c_{H_2O}$ were determined through a wide range of solute excretion (mannitol and/or sodium chloride) in normal dogs receiving infusions of vasopressin. A characteristic curve was observed through which values for $T^c_{H_2O}$ increased to a maximum and then diminished, with further increases in solute excretion. It is suggested that diminishing values for $T^c_{H_2O}$ may result from failure of complete passive osmotic equilibration in the distal convolution at very high rates of solute excretion. Immediate comparison of values for $T^c_{H_2O}$ at various rates of osmolal clearance with and without chlorothiazide were obtained. The values for $T^c_{H_2O}$ at low rates of osmolal clearance during the administration of chlorothiazide did not differ from values obtained without the drug. At higher rates of osmolal clearance, $T^c_{H_2O}$ during the infusion of chlorothiazide often exceeded the maximal control value, and this agent uniformly resulted in increased values for $T^c_{H_2O}$ at rates of osmolal clearance which, in the absence of the drug, were associated with a decreasing $T^c_{H_2O}$.

Changes in the rate of glomerular filtration during the infusion of chlorothiazide were not uniform and were not always associated qualitatively with the observed changes in C_{H_2O} and $T^c_{H_2O}$.

It is suggested that these data are consistent with the premise that chlorothiazide increases electrolyte excretion by interfering with its reabsorption in portions of the nephron where the tubular fluid is normally diluted. The failure of this agent to lower $T^c_{H_2O}$ suggests that this action takes place predominantly in the distal convolution rather than in the ascending limb of Henle's loop.

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SODIUM DEPLETION AND RENAL CONSERVATION OF WATER*

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The deleterious influence of sodium depletion upon circulatory efficiency and renal function is well known (1–5). Glomerular filtration rate and renal blood flow are generally decreased, sodium excretion declines to the vanishing point, and the ability to excrete a water load is impaired (4, 6). Dilutional hyponatremia, by contrast, is often attended by an increase in glomerular filtration rate and enhanced excretion of sodium (7, 8), as well as by some decline in maximal urinary concentration of solutes (9).

The present experiments, carried out on rats, were designed to explore the effect of hyponatremia induced by the removal of body sodium upon the ability of the kidneys to conserve water and to concentrate the urine maximally. Maximal urinary concentration was not diminished by merely removing sodium from the diet. However, rats depleted of sodium by peritoneal dialysis demonstrated a considerable fall in renal concentrating ability which resulted in striking losses of water from the body and eventual restoration of serum sodium at the expense of the volume of body fluids.

METHODS

Group I. The effect of a sodium-free diet upon renal concentrating ability. Eight male Sprague-Dawley rats weighing 240 to 290 g were fed a synthetic diet containing liberal amounts of sodium and were allowed food and water *ad lib.* for 6 days. At the end of this time, food and water were withheld and 100 mU of vasopressin suspended in peanut oil was injected subcutaneously. Urine was collected under mineral oil for 24 hours and its osmolality (U_{max}) was determined with a Fiske osmometer. The procedure was repeated on the same animals after they had received a diet containing no sodium for 6 days.

Twelve additional rats, weighing 290 to 310 g, were fed a sodium-free diet for 6 days, at the end of which U_{max} was determined. A diet containing adequate amounts of sodium was then allowed but, in order to keep the intake of protein and calories constant, only in amounts limited to those the same animal had consumed during its preceding sodium-free period. In this way each rat served as its own pair-fed control. After 6 days U_{max} was again measured.

Group II. The effect of severe hyponatremia upon renal concentrating ability. Nineteen rats (group IIa), weighing 375 to 425 g, were placed on a sodium-free diet. Subsequently a stainless steel cannula of the type described by Kolff and Page (10), was sewn into the peritoneal cavity of each animal and the operative incision allowed to heal. The rats were then placed in individual metabolism cages so that the intake of food and water and the output of urine could be accurately measured. After 2 days of control observations they were subjected to peritoneal dialysis. The dialysis fluid consisted of 5 per cent glucose in water containing 25 mEq per L of $NaHCO_3$. (The latter was included to minimize acidosis as a result of losses of bicarbonate from the body during dialysis.) A volume of warmed fluid equal to 10 per cent of the body weight was instilled through the cannula into the peritoneal cavity and withdrawn after 2 hours. The procedure was then repeated. The amount of dialysis fluid removed at the end of the 4 hour period was approximately equal to that instilled. At the conclusion of the dialysis, serum sodium, blood urea nitrogen and hematocrit were determined on 0.5 ml of tail blood. Balance studies were resumed on the following morning. Following dialysis, 8 rats were given daily subcutaneous injections of 100 mU of vasopressin in oil for the remainder of the experiment.

Eight days after dialysis, the animals were sacrificed by aortic exsanguination. The shafts of both femurs were removed, cleaned of marrow and, after drying, analyzed for sodium using a Baird flame photometer. Samples of thigh muscle were dried, extracted with anhydrous ether and analyzed for sodium. Both kidneys were removed, fixed in formalin and examined for morphological changes. Hematocrit, serum sodium, serum CO_2 content and blood urea nitrogen (11) were determined. Daily urine samples were analyzed for sodium, calcium (12), urea or total nitrogen, and osmolality.

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TABLE I

Effect of a low sodium diet upon maximal urinary osmolality

	Low sodium diet		Regular diet		P (Low sodium vs regular)
	Food intake	U_{osm}	Food intake	U_{osm}	
	g	*mOsm/kg*	*g*	*mOsm/kg*	
Group Ia n = 8	27.5* ±1.8	2,394 ±314	33.1 ±3.6	2,730 ±274	<0.01
Group Ib n = 12	29.6 ±4.4	2,376 ±247	29.6 ±4.4	2,227 ±200	N

* Mean ±SD.

Thirteen rats (group IIb) were handled in exactly the same manner as those of group II, save that their dialyzing fluid consisted of 5 per cent glucose containing 140 mEq per L of Na, 115 mEq per L of Cl and 25 mEq per L of HCO_3. Following dialysis, dietary intake of these control animals was limited to the average daily intake of the hyponatremic rats of group IIa.

Group III. The effect of moderate hyponatremia upon renal concentrating ability. Eight rats were treated in the same manner as group IIa, except that the second peritoneal dialysis was omitted.

RESULTS

Effect of a low-sodium diet upon renal concentrating ability (groups Ia and b, Table I). Maximal urinary osmolality decreased slightly when rats allowed to eat *ad lib.* were transferred from a regular to a salt-free diet (group Ia). Pair-feeding experiments (group Ib) indicated that this was the result of diminished food intake rather than an effect of salt restriction per se on the kidney. When the intake of animals eating a regular diet was limited to that ingested on a sodium-free diet, no difference in U_{max} was apparent. Serum sodium was unaltered by a low-sodium diet.

Effect of severe hyponatremia upon renal concentrating ability (groups IIa and b, Tables II and III). As a result of sodium depletion by dialysis, serum sodium fell to an average of 107 mEq per L. Blood urea nitrogen rose to an average of 53 mg per 100 ml and the hematocrit was elevated. Serum sodium, blood urea and hematocrit remained normal in rats dialyzed against a solution containing 150 mEq per L of sodium (group IIb).

Following dialysis there was a precipitous weight loss in the hyponatremic animals, amounting by the seventh day to 22 per cent of the original weight. By this time, when the rate of weight loss had diminished and body weight had begun to plateau, the average serum sodium had risen from 107 to 139 mEq per L. Weight loss was not so marked in the control animals, whose serum sodium remained unaltered throughout the experiment (Table II, Figure 1).

Immediately following dialysis, urinary output increased markedly in the hyponatremic animals

TABLE II

Effect of hyponatremia upon weight and body composition

	Weight		Serum Na		BUN			Hematocrit
	Predialysis	Final	Postdialysis	Final	Postdialysis	Final	Bone Na	Postdialysis
	g		*mEq/L*		*mg %*		*mEq/100 g dry solids*	*%*
Group IIa								
Without vasopressin n = 6	398 ±22	320 ±18	111 ±13	139 ± 8	51 ±6	49 ±45	0.273 ±0.011	56 ±1
With vasopressin n = 8	399 ±18	310 ±24	106 ± 6	139 ± 9	55 ±7	44 ±23	0.275 ±0.009	53 ±6
Group IIb								
Without vasopressin n = 6	396 ±22	348 ±21	147 ± 3	150 ± 1	19 ±5	15 ± 1	0.286 ±0.006	48 ±2
With vasopressin n = 7	397 ±22	344 ±18	152 ± 7	147 ± 2	19 ±3	13 ± 3	0.291 ±0.009	47 ±1
P IIa vs IIb No vasopressin	NS	0.01	0.01	0.01	0.01	0.01	0.10	0.01
With vasopressin	NS	0.01	0.01	0.05	0.01	0.01	0.01	0.05

TABLE III

Effect of hyponatremia upon urinary concentration and solute excretion

Day	−2		−1	0*	+1		+2		+3		+4		+5		+6	
	U_{osm}	$U_{osm}V$	U_{osm}	$U_{osm}V$	U_{osm}	$U_{osm}V$	U_{osm}	$U_{osm}V$	U_{osm}	$U_{osm}V$	U_{osm}	$U_{osm}V$	U_{osm}	$U_{osm}V$	U_{osm}	$U_{osm}V$
	mOsm/kg	*mOsm*	*mOsm/kg*	*mOsm*	*mOsm/kg*	*mOsm*	*mOsm/kg*	*mOsm*	*mOsm/kg*	*mOsm*	*mOsm/kg*	*mOsm*	*mOsm/kg*	*mOsm*	*mOsm/kg*	*mOsm*
Group IIa																
Without vasopressin n = 11	1,939 ±418	19.5 ±4.9	1,918 ±648	21.2 ±6.3	470 ± 95	13.9 ±4.4	742 ±107	16.7 ±2.7	985 ±208	18.2 ±4.1	1,105 ±289	15.7 ±4.3	918 ±209	10.8 ±4.0	977 ±371	13.4 ±3.9
With vasopressin n = 8	1,764 ±374	17.8 ±2.5	1,975 ±506	21.0 ±3.3	598 ±169	14.8 ±4.0	814 ±158	15.7 ±3.7	1,110 ±224	18.4 ±3.6	1,291 ±341	16.6 ±4.0	972 ±194	11.2 ±3.9	1,088 ±499	13.0 ±3.5
Group IIb																
Without vasopressin n = 6	2,287 ±179	18.8 ±2.5	2,408 ±229	20.0 ±3.9	1,853 ±343	9.2 ±2.0	1,432 ±512	9.8 ±2.6	1,842 ±499	10.5 ±2.8	1,937 ±320	8.9 ±1.1	1,892 ±628	11.6 ±4.4	2,288 ±674	12.5 ±3.7
With vasopressin n = 7	2,241 ±245	19.4 ±2.3	2,146 ±334	22.5 ±8.1	1,694 ±451	8.7 ±0.8	1,460 ±381	7.6 ±1.4	1,780 ±547	9.5 ±3.5	1,651 ±493	8.9 ±3.4	1,905 ±563	13.9 ±2.6	2,237 ±674	13.9 ±1.9

* Dialysis.

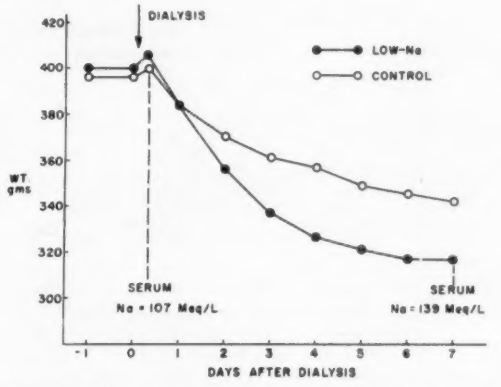

FIG. 1. BODY WEIGHT FOLLOWING THE INDUCTION OF HYPONATREMIA. Hyponatremic rats lost weight rapidly until serum sodium approached normal levels, one week after dialysis.

(Figure 2). Food intake decreased. The intake of water diminished slightly, although not so much as in comparably-fed controls. Total solute excretion was depressed. Sodium excretion remained negligible, the urinary concentration of sodium being less than 1 mEq per L. The diuresis was associated with a striking fall in urinary osmolality (Table III, Figure 3), which declined from 1,900 mOsm per kg before dialysis to 500 mOsm per kg after the procedure. Urinary osmolality rose slowly over the next several days as diuresis and weight loss became less pronounced and the serum sodium approached normal levels. A slight fall in urinary osmolality was also observed in the control rats, presumably as a result of their restricted food intake.

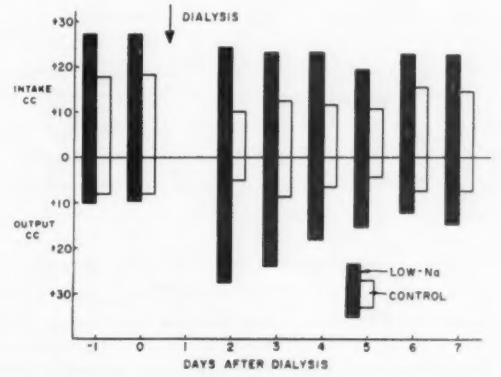

FIG. 2. URINARY OUTPUT AND WATER INTAKE FOLLOWING HYPONATREMIA.

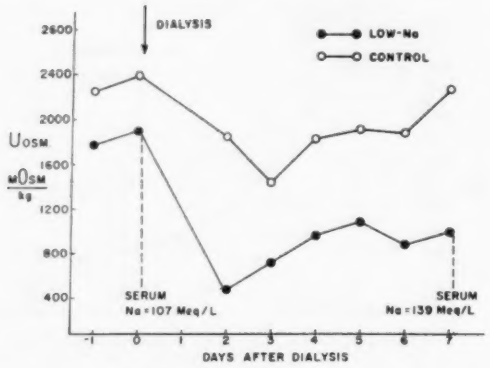

FIG. 3. URINARY OSMOLALITY FOLLOWING HYPONATREMIA.

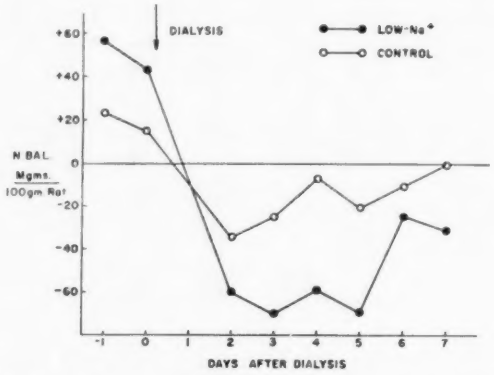

FIG. 5. EFFECT OF HYPONATREMIA UPON NITROGEN BALANCE. Animals depleted of sodium developed a negative nitrogen balance which was more pronounced than that observed in pair-fed controls.

The diuresis following sodium depletion was not altered by the daily administration of vasopressin in oil (Figure 4). Vasopressin prevented neither the weight loss nor the marked drop in urinary osmolality associated with hyponatremia. Similar experiments were performed, in which 1,000 mU of aqueous vasopressin was injected subcutaneously every 2 hours for 48 hours after the production of hyponatremia. The postdialysis diuresis was unaffected.

As suggested by other reports (1, 13, 14), animals depleted of sodium developed a negative nitrogen balance which was more pronounced than that observed in controls (Figure 5). Net urinary losses of potassium in the two groups was similar and proportional to net losses of nitrogen

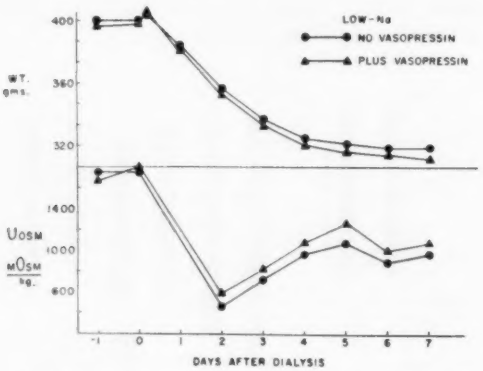

FIG. 4. THE EFFECT OF EXOGENOUS VASOPRESSIN UPON WEIGHT LOSS AND DECREASED URINARY SOLUTE CONCENTRATION ASSOCIATED WITH HYPONATREMIA. Urinary losses of water were not prevented by the administration of vasopressin to hyponatremic rats.

in the approximate ratio of 3 mEq of potassium to 1 g of nitrogen.

Bone sodium (Table II) was slightly lower in the sodium-depleted animals than in their controls; the losses of sodium from bone, however, were not nearly so striking as those observed in sodium depletion associated with acidosis (15). Urinary calcium excretion and serum calcium levels were unaffected by dialysis, further suggesting that sodium depletion did not result in dissolution of bone. The average muscle sodium in depleted rats at the time of sacrifice was 7.65 mEq per 100 g fat-free dry solids (FFDS), compared with 8.23 mEq per 100 g FFDS in control animals.

Microscopic examination of the kidneys showed severe hydropic changes in the proximal tubules of all animals, with similar though less marked alterations in the distal convoluted tubules. Droplets of eosinophilic material were apparent in the tubular cells in these locations, and eosinophilic material was also present in the lumens of the collecting ducts. This material did not have the staining characteristics of a mucoprotein. Glomeruli, vessels and collecting ducts appeared otherwise normal. The changes in cortical tubules resembled those described by Hamburger, Halpern and Funck-Brentano (16) in rabbits with dilutional hyponatremia.

Effect of moderate hyponatremia upon renal concentrating ability (group III, Table IV). After a single dialysis, average serum sodium fell to

TABLE IV

Effect of moderate sodium depletion upon urinary concentration

	Weight		Serum Na		BUN		U_{max}		
	At dialysis	After 7 days	After dialysis	After 7 days	After dialysis	After 7 days	Before dialysis	Day 1 after dialysis	Day 7 after dialysis
	g		*mEq/L*		*mg %*		*mOsm/kg*		
Group III, 8 Na-depleted rats	392 ±23	340 ±36	135 ±0.2	159 ±2	50 ±11	24 ±7	2,324 ±354	885 ±168	2,604 ±451

135 mEq per L and blood urea nitrogen rose to 50 mg per 100 ml. Maximal urinary osmolality declined, diuresis and weight loss ensued and serum sodium rose, in a manner entirely similar to that observed in the more severely hyponatremic rats of group IIa.

DISCUSSION

Hyponatremia produced in the rat by depletion of sodium results in a diuresis of urine of relatively low osmolality and negligible sodium concentration, which continues until the serum sodium is returned to normal. The increase in urine flow is not the result of an osmotic diuresis, since solute excretion actually decreases. Nor is it a consequence of diminished secretion of antidiuretic hormone. It is not prevented by injections of vasopressin and is clearly a reflection of a decrease in the ability of the kidneys to concentrate the urine.

A similar decrease in maximal urinary concentration has been observed in *dilutional* hyponatremia in dogs (9) and man (17). Under these circumstances, there is frequently an increase in glomerular filtration rate or total solute excretion. In the present study, glomerular filtration rate was undoubtedly diminished by sodium depletion, and solute excretion was uniformly decreased. *Acute* decreases in glomerular filtration reduced maximal urinary concentration in dogs studied by Leaf, Kerr, Wrong and Chatillon (18) and Levinsky, Davidson and Berliner (19). This was associated, however, with a decrease in urine flow, rather than the sustained increase in urinary output which we observed in hyponatremic rats. Other causes of impaired concentrating ability, such as potassium depletion, hypercalcemia and hypercalciuria, were excluded in the present experiments. Lowering the concentration of urinary sodium per se, by feeding a low salt diet, does not appear to change maximal urinary concentration of the rat, in the absence of hyponatremia.[1]

The mechanism of the decrease in concentrating ability induced by hyponatremia is not clear. It seems probable that the interstitial fluid of the renal medulla and papilla of hyponatremic rats is less highly concentrated in sodium than that of normal animals, either because of changes in renal medullary blood flow which reflect profound alterations in the systemic circulation, or because of a depression of active sodium transport by the medullary tubules.

The degree of impairment of renal concentrating ability and its net effect upon body water and the concentration of serum sodium may vary from one species to another and even among individuals. An abrupt diuresis, loss of weight, and rise in serum chloride were observed by Semple in rats depleted of electrolytes by dialysis (22). Darrow and Yannet (13) depleted three dogs of sodium by peritoneal dialysis; in one the serum sodium subsequently rose spontaneously from 129 to 140 mEq per L while it was on a salt-free diet, but hyponatremia persisted in the others. In a similar experiment in a dog, reported by Leaf and Mamby (23), serum sodium also remained low and body weight was undiminished. Holmes and Cizek (24) and Cizek, Huang, Semple and Gregersen (6, 25) noted an increase in water intake and urinary output in sodium-depleted dogs; serum chloride, however, remained low when the animals were fed a low salt diet after dialysis. Hyponatremia induced in humans by sodium removal over several days commonly persists until salt is supplied (1). We are unaware of any detailed study of the effect of sodium

[1] Maximal urinary concentration of dogs (20) and humans (21) is similarly unaffected by a diet low in sodium.

depletion upon renal concentrating ability in human subjects.

It is of interest that the intake of water remained high in sodium-depleted rats even though extracellular tonicity was low, a state of affairs that might have been expected to reduce thirst (*26*). Presumably, thirst was sustained under these circumstances by a response to contraction of the volume of extracellular fluid and blood or to other alterations in the circulation which may have been caused by hyponatremia.

Restoration of the concentration of sodium in extracellular fluid was accomplished almost entirely by losses of water from the body. The contribution of bone sodium to replacement of sodium deficits was negligible. This finding contrasts with studies in which sodium depletion was associated with acidosis (15) but is in accord with other experiments in rats in which (as in the present study) acidosis was minimized by placing sodium bicarbonate in the solution used for peritoneal dialysis (*27*).

In addition to other implications, these experiments provide an interesting physiological example of at least one situation in which tonicity is preserved at the expense of body fluid volume. Rats apparently respond to severe hyponatremia by literally shrinking around their available sodium. This does not appear, however, to result from an adaptive reaction of the hypothalamic centers concerned with thirst and the secretion of antidiuretic hormone. It is rather a consequence of severe, reversible impairment of the renal mechanism for concentrating urine and conserving water.

SUMMARY

1. Rats depleted of sodium and rendered hyponatremic by peritoneal dialysis develop severe, reversible impairment of renal concentrating ability, resulting in increased urinary losses of water, rapid weight loss and eventual restoration of serum sodium to the normal range.

2. The contribution of bone sodium to replacement of sodium deficits under these circumstances is negligible.

3. Removal of sodium from the diet does not change maximal urinary concentration in the rat if serum sodium is unaltered and protein intake is kept constant.

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ISOAGGLUTININS ASSOCIATED WITH ABO ERYTHROBLASTOSIS *

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The occurrence and severity of erythroblastosis fetalis, due to maternal Rh isoimmunization, can be predicted through simple prenatal serological tests (1, 2). If Rh isoagglutinins are present in maternal serum, and if the incompatible Rh antigen is transmitted from the father, the newborn will have hemolytic disease, the severity of which is often proportional to the titer of the mother's serum Rh isoagglutinins (1, 2). In the event that the isoimmunized Rh negative mother delivers an Rh negative infant, the titer of Rh antibodies in the cord serum, except for saline agglutinins, is generally equal to the titer obtained in the maternal serum (3). For practical purposes, almost all maternal Rh isoagglutinins seem to cross the placental barrier freely, and their titration value affords guidance toward the effective management of hemolytic disease of the newborn. This has not been the case for erythroblastosis fetalis due to ABO incompatibility (1, 2).

ABO erythroblastosis of sufficient severity to warrant exchange transfusion therapy for the control of hyperbilirubinemia, has a frequency equal to that of erythroblastosis due to Rh incompatibility (2, 4), and yet prenatal tests have been of little value thus far in identifying those mothers who may deliver affected infants (4). Since ABO erythroblastosis occurs often in the first incompatible pregnancy (5, 6), a reliable prenatal test would be of extreme importance in anticipating the occurrence of this disease for intelligent management.

A varying proportion of maternal anti-A (α) and anti-B (β) isoagglutinins can be demonstrated in the serum of the newborn (7, 8). Most ABO isoagglutinins cannot cross the placenta and therefore play no role in the pathogenesis of erythroblastosis. Fudenberg, Kunkel and Franklin (9) suggested that 7S isoagglutinins traverse the placental barrier, thus predisposing to hemolytic disease. If so, diffusion of antibodies across the placenta may be dependent upon the molecular size of the antibody molecule, with low molecular weight (about 160,000) γ_2 antibodies of the mother reaching the fetus (9).

Of equal importance in the pathogenesis of ABO hemolytic disease of the newborn are the protective effects of nonerythrocytic ABO blood group substances in the fetus. Whereas Rh antigens appear to be restricted to the red blood cells, ABO antigens occur in other tissues, as well as in the secreted mucopolysaccharides of most persons (10), and ABO isoagglutinins possess varying degrees of sensitivity to inhibition by specific soluble blood group substances (SSBGS) (11, 12). An excess of ABO secretors has been observed in infants with ABO erythroblastosis (5, 6, 13, 14) and in their fathers (14). This excess may reflect either a sensitivity of ABO secretors, or a resistance of ABO nonsecretors, toward ABO disease, or even a loss of ABO nonsecretors *in utero*.

Since blood group substances are readily demonstrable in the tissues of both secretors and nonsecretors (15), further quantitative and/or qualitative distinctions must exist between these two classes to explain these observations. Resistance to neutralization of some α and β isoagglutinins might be related to the clinical problem of ABO erythroblastosis, if such antibodies were 7S γ_2-globulins crossing the placenta (9). Large antibody molecules (macroglobulins), because they fail to traverse the placental barrier, are not pertinent to this problem (9).

Column chromatographic separation of serum proteins on anion exchanger, diethylaminoethyl (DEAE) cellulose, first described by Sober, Gutter, Wyckoff and Peterson (16, 17), offers a relatively simple means of separating 7S γ_2-globulins from other serum proteins (18). Such separation of human isoagglutinins has been reported by

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Abelson and Rawson (19) and by Fahey and Morrison (20).

The present report deals with the resistance to neutralization by SSBGS of ABO isoagglutinins which can traverse the placental barrier. These isoagglutinins were obtained by a simplified DEAE cellulose column chromatographic method, designed to separate 7S γ_2-globulins from the remainder of the serum proteins. The results observed in the presence and the absence of ABO erythroblastosis suggest that the disease might be anticipated by suitable prenatal tests.

MATERIALS AND METHODS

Chromatographic separation was performed according to a modified method of Sober and Peterson (17) and of Levy and Sober (18). In general, the following procedure was used for separation of serum: DEAE cellulose [1] was purified according to Sober and co-workers (16) and the slurry, suspended in the initial buffer (0.02 M phosphate, pH 6.3), was poured into 5-ml serological pipets (25 × 0.5 cm). The ion exchanger was packed under water pressure of 40 to 50 cm and washed with the initial buffer overnight. The dead volume of such columns was about 2.5 ml.

The serum sample, dialyzed [2] against 3 changes of the initial buffer in a 4° C cold room, was applied to the column, together with the slight precipitate formed during dialysis. For each separation, 1 ml serum (corrected for the increase of volume during dialysis) was applied to the column at room temperature, and washed with 10 ml of the initial buffer, followed by 10 ml of 1 M NaCl. Two-ml samples were collected at a flow rate of 6 to 8 ml per hour, at room temperature. The protein content of the separated fractions was measured by absorption at 280 mμ in a Beckman DU spectrophotometer.

Under these conditions, the 0.02 M phosphate buffer (pH 6.3) fraction (hereafter referred to as 0.02 M fraction) contained the first four chromatographic peaks described by Humphrey and Porter (21), and if isoagglutinins were present in this fraction, the highest titers were always found in those tubes which contained most protein. No difference was found in the elution pattern of the isoagglutinins when 0.01 M phosphate buffer was followed by 0.02 M buffer, or when more than 10 ml of buffer was used as first eluent (Figure 1). The 0.02 M fraction was not found to contain proteins other than γ_2-globulins when tested by paper electrophoresis and acrylamide electrophoresis (22). Ultracentrifugation of this fraction at a protein concentration of 1 per cent (courtesy of Drs. O. Singher and R. Fenichel, Ortho Research Foundation, Raritan, N. J.) revealed only a single component having a sedimentation constant of 7S. Heavier components were not observed despite special attention for their detection in the earliest parts of the ultracentrifugal run.

The second fraction eluted with 1 M NaCl (hereafter referred to as 1 M fraction) contained 19S macroglobulins and all other serum proteins, but paper and acrylamide electrophoresis failed to reveal γ_2-globulins.

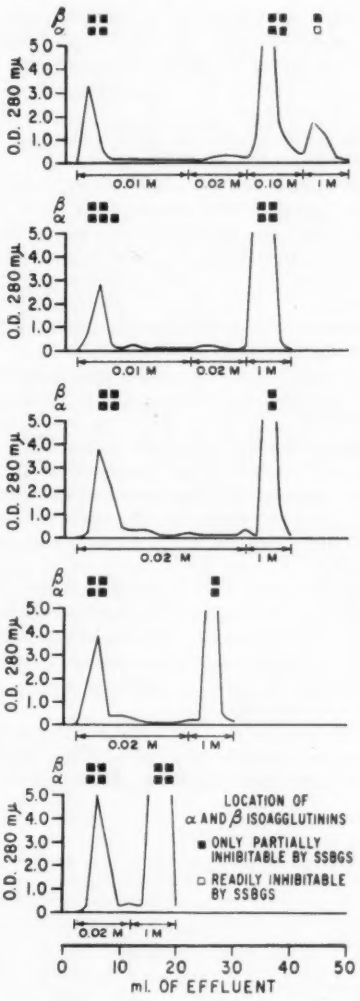

FIG. 1. SEPARATION OF ISOAGGLUTININS ON DEAE CELLULOSE COLUMNS UNDER VARYING CONDITIONS. Variations of the concentration (0.01 to 0.02 M) of the phosphate buffer, pH 6.3, and of the volume of effluent collected (10 to 30 ml), failed to alter the pattern of 7S γ_2-globulins or α and β isoagglutinins emerging from the column. The α and β isoagglutinins in the fractions eluted with 1 M NaCl could be further separated by using 0.1 M phosphate buffer, pH 6.3, prior to 1 M NaCl.

[1] Eastman Organic Chemicals, Rochester, N. Y.

[2] Cellulose casings, Visking Co., Chicago, Ill.

The isoagglutinin activity of this fraction could be split into two fractions using, after 0.02 M phosphate buffer, pH 6.3, first 0.1 M phosphate buffer, pH 6.3, followed by 1 M NaCl (Figure 1). The significance of this observation is under study.

The isotonicity of 0.02 M phosphate buffer fractions was restored by addition of 0.05 ml of 18 per cent NaCl per 1 ml of eluate, and of 1 M NaCl fractions by dilution with 5 vol of distilled water.

Native serum and pooled fractions, the latter concentrated to the original serum volume by dialysis at 4° C against 20 per cent polyvinylpyrrolidone (PVP), provided materials for titration of antibodies before and after separation. Parallel titrations showed a good recovery of the isoagglutinins (Table I).

The agglutination tests for α and β isoagglutinins were done by conventional methods, usually in duplicate. Twofold serial dilutions of serum or fractions were mixed with equal volumes of 2 per cent washed red cells from type A_1 and B donors. After incubation at 37° C for 1 hour and centrifugation, agglutination was recorded as the saline titer. An additional volume of 4 per cent acacia (5) was then added and the mixture reincubated for 15 minutes at 37° C, centrifuged, and 1 vol of saline added to abolish rouleaux. The agglutination then observed, usually by two observers, neither familiar with the clinical details, was recorded as the acacia titer. This concentration of acacia was chosen because it afforded results which agreed within one dilution of those observed with the γ-antiglobulin test.

The neutralization tests for α and β isoagglutinins were performed by mixing serial dilutions of 0.02 M phosphate buffer fractions with suitable serial dilutions of SSBGS of hog (A) and horse (B) origin.[3] The test cells were added after 15 minutes' incubation at room temperature. After further incubation for 1 hour at 37° C and centrifugation, agglutination was recorded as saline titer. Then 4 per cent acacia was added to the tests which were further incubated for 15 minutes at 37° C. The agglutination observed after centrifugation and addition of saline was recorded as the acacia titer. The acacia titer was always found to be more reliable and is given throughout this report. Titers with and without SSBGS were frequently checked for reproducibility at a later date and consistent results were always obtained.

TABLE I

Recovery of ABO isoagglutinins after fractionation on DEAE cellulose columns

Serum no.	Antibody	Isoagglutinin titers (reciprocal)			
		Native	0.02 M*	1 M†	Pool‡
3	α	80	0	80	80
	β	160	0	80	80
21	α	1,280	160	640	1,280
	β	640	40	320	640
42	α	640	80	320	640
	β	160	4	80	80
140	α	160	4	80	80
	β	160	0	160	160

* Titer of pooled fractions that were eluted with 0.02 M phosphate buffer, pH 6.3, and concentrated to original serum volume.

† Titer of pooled fractions that were eluted with 1 M NaCl and concentrated to original serum volume.

‡ Mixture of * and † corrected for volume change due to mixing.

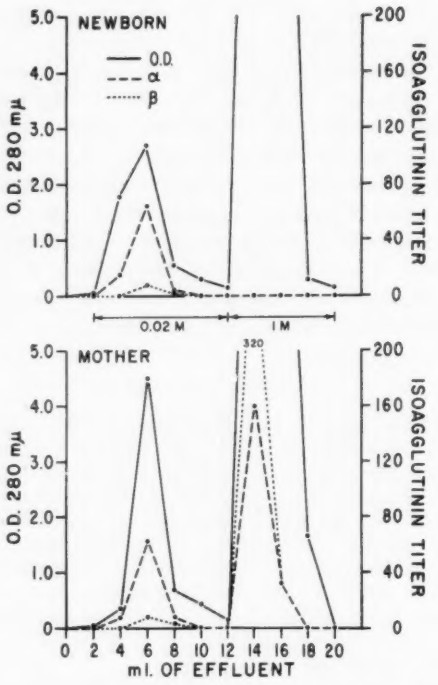

FIG. 2. THE RECOVERY OF MATERNAL AND FETAL ABO ISOAGGLUTININS FROM DEAE CELLULOSE COLUMNS. Both mother (no. 268) and newborn (no. 265) type O; 1 ml serum samples fractionated. Isoagglutinin titers of whole serum: mother, α 1:512 and β 1:512; newborn, α 1:160 and β 1:16. Isoagglutinin titer in figure is represented by reciprocal values.

RESULTS

1. The separation of cord serum α and β isoagglutinins on DEAE cellulose columns. ABO isoagglutinins have been demonstrated in cord bloods, especially those from type O offspring of type O mothers. Cord serum ABO isoagglutinins could

[3] Biosales Inc., 300 W. 43rd St., New York, N. Y.

TABLE II

*The recovery of cord blood * isoagglutinins in 0.02 M eluates † from DEAE cellulose columns ‡*

Specimen no.	Blood type		Isoagglutinin titer (reciprocal)			
			Native cord serum		0.02 M fraction	
	Mother	Child	Anti-A	Anti-B	Anti-A	Anti-B
44	O	O	160	10	40	<10
62	O	O	80	160	40	40
117	O	O	40	<10	20	<10
143	O	O	80	<10	40	<10
144	O	O	20	<10	20	<10
145	O	O	160	<10	80	<10
146	O	O	80	40	80	20
147	O	O	160	<10	80	<10
148	O	O	40	40	40	40
150	O	O	320	10	160	<10
152	O	O	80	10	40	<10
153	O	B	20		20	
154	O	O	20	<10	<10	<10
155	O	O	40	20	40	20
156	O	O	80	<10	80	<10
180	O	B	80		40	

* Cord sera selected to have >1:10 anti-A.
† Cord blood isoagglutinins not observed in the 0.1 to 1.0 M eluates of these and 17 other cord sera tested.
‡ Unconcentrated single 0.02 M phosphate buffer, pH 6.3, eluates which contained the most isoagglutinins were titrated and the values adjusted for dilution over the volume of the native serum.

be of 7S character (9), because they are incomplete reflections of α and β isoagglutinins present in the serum of the mother.

Thirty-three cord sera, each containing α and β isoagglutinins, were separated on DEAE cellulose columns, and eluates collected as described under Methods. The isoagglutinins of these sera were recovered only in the 0.02 M fractions; the 1 M fractions, under the standard test conditions, did not reveal isoagglutinin activity (Figure 2). Rarely, and only when some cord sera contained in excess of 1:200 titer of ABO isoagglutinins, concentrations of 1 M NaCl fractions contained irregular trace amounts of isoagglutinins up to approximately 1 per cent of the native titer, due perhaps to contamination with 7S γ_2-globulins.

Sixteen sera were found to have anti-A in a titer of 1:20 or greater, and these sera were studied by titration of the native material and that single 0.02 M fraction which contained most of the isoagglutinin activity. There was no need to pool or to concentrate fractions, because these antibodies invariably appeared with the effluent having most of the 7S γ_2-globulins, and one fraction always contained most of the isoagglutinins in a titer quite similar to that of the native cord serum (Figure 2). Titrations were seldom performed in parallel, and the test red cells were sometimes varied. Table II summarizes these results and shows the good recovery of cord serum agglutinins in single 0.02 M fractions from DEAE cellulose columns.

2. Comparison of the 7S α and β isoagglutinin titers of maternal and cord sera. If the α and β isoagglutinins of cord sera are 7S γ_2-globulins that can be recovered in 0.02 M fractions from DEAE cellulose columns, and if these isoagglutinins are of maternal origin, having diffused across the placental barrier, the 0.02 M fractions of maternal and cord serum pairs (collected at the time of delivery) should be expected to have identical titer values, if equilibrium is attained.

Eight pairs of maternal and cord sera, all type O and containing α and β isoagglutinins, were fractionated on DEAE cellulose columns. The 0.02 M fractions and the native maternal sera were titrated for α and β isoagglutinins, and the results (summarized in Table III) show that the 0.02 M fraction of the α and β isoagglutinin activity in maternal serum is duplicated in the cord serum. The quantitative data of one of these maternal-cord pairs, shown in Figure 2, reveals that these isoagglutinins, as well as other 7S γ_2-globulins without isoagglutinin activity, exist in equilibrium on both sides of the placenta.

3. The occurrence of α and β isoagglutinins in the 0.02 M fractions of 110 prenatal specimens. Study of the cord sera of compatible pregnancies (7) has furnished some information concerning placenta-crossing ABO isoagglutinins, but there has been no method thus far to study such antibodies in unselected pregnancies in advance of delivery. The 0.02 M fractions of DEAE cellu-

TABLE III

*Comparison of the α and β isoagglutinin titers * of maternal and cord sera obtained at delivery †*

Specimen no.	Isoagglutinin titer (reciprocal)					
	Infant 0.02 M fraction		Maternal 0.02 M fraction		Native maternal serum	
	α	β	α	β	α	β
26/45	160	80	160	160	640	640
56/61	40	4	40	20	160	160
27/63	20	<2	20	<2	80	80
107/119	80	40	40	80	320	640
135/43	20	20	20	20	160	160
139/157	20	40	20	40	160	160
169/171	40	2	40	4	640	80
265/268	64	8	64	8	512	512

* Unconcentrated single 0.02 M phosphate buffer, pH. 6.3, eluates which contained the most isoagglutinins were titrated, and the values adjusted for dilution over the volume of the native serum.
† Mothers and newborn all type O.

TABLE IV

The distribution of the ABO blood types of the newborn of 110 mothers of types O, A and B

Blood type, newborn	Blood type, mother		
	O	A	B
O	45	5	7
A	20	20	0
B	8	1	1
AB	0	2	1
Total	73	28	9

lose columns offered such an opportunity, and 110 prenatal specimens of type O, A and B patients were fractionated. Table IV shows the distribution of the blood types of the newborn subsequently delivered, and Table V reveals the distribution of the α and β isoagglutinins in maternal 0.02 M fractions. To be detected by the screening tests employed, a titer of at least 1:2 was required.

In this series, isoagglutinins were not observed in the 0.02 M fractions of the sera of 28 type A mothers and 9 type B mothers, but were observed in the sera of 53 out of 73 type O mothers. Deviation from our earlier results in study of compatible gestation (7) may be due to the dilution at which these tests were now performed. Whereas the cord serum of compatible newborn can be tested without dilution, the unconcentrated 0.02 M fractions represented a dilution of at least 1:2 over the volume of the native serum that was placed on the cellulose columns. A further slight dilution occurred when concentrated NaCl was added for isotonicity, and some loss of antibody was expected, because only one of several antibody-containing, or potentially antibody-containing, fractions was tested. This small study of 0.02 M fractions revealed only that ABO isoagglutinins in a titer of at least 1:2 were observed in 69 per cent of 73 type O mothers and were not observed in 37 mothers of type A or B. This is consistent with the strong association of ABO erythroblastosis with mothers of type O (16).

TABLE V

The occurrence of α and β isoagglutinins of at least 1:2 titer in the 0.02 M phosphate buffer fractions of 110 mothers of blood types O, A and B

	Blood type, mother		
	O	A	B
$\alpha + \beta$	24	0	0
α only	27	0	0
β only	2	0	0
No isoagglutinins	20	28	9

Furthermore, it is of interest to note (Table V) that anti-B was observed in only 26 of 73 type O mothers, whereas anti-A was found in 51 of the same mothers. In addition, β without α was observed only twice, but α without β was encountered 27 times. This preponderance of α activity might well be associated with the preponderance of erythroblastosis due to A incompatibility (7).

4. Relative inhibition by specific soluble blood group substances of the isoagglutinins in the 0.02 M fractions of 53 type O mothers. Table V reveals that the placenta-crossing α and β isoagglutinins contained in 7S γ_2-globulin fractions occur commonly, but clinical ABO erythroblastosis is observed in only 1 of 200 newborn (23). This difference could be due to the protective effect of extraerythrocytic ABO antigens.

Witebsky (11) suggested that "non-inhibitable" ABO isoaglutinins might be related to ABO erythroblastosis, but Zuelzer and Kaplan (6) noted a poor correlation. However, the study of total serum isoagglutinins (12) would not be informative, since the bulk of α and β antibodies are 19S (20) (Table V), which not only fail to cross the placental barrier, but could confuse the results.

The erythrocytic, tissue, and mucopolysaccharide ABO antigens, even of animal origin, share common structural groupings. On the other hand, agglutinins which are resistant to neutralization by large quantities of SSBGS permit a distinction between erythrocytic and extraerythrocytic ABO antigens. Therefore, diffusible noninhibitable maternal isoantibodies, even of low titer, could be responsible for clinical ABO erythroblastosis. The 0.02 M cellulose column fractions of the isoagglutinins of 53 type O mothers, presented an opportunity to attack this problem directly.

Titration of the ABO isoagglutinins in the 0.02 M fractions of these 53 mothers yielded values ranging from 1:4 to 1:640, and little if any correlation with the native serum titers (Table II). Of more importance were duplicate titrations to which SSBGS were added to each tube of the titration series. The concentration of the SSBGS was varied from 1:10 to 1:500 for A substance, and from 4:1 to 1:500 for B substance, to deter-

TABLE VI

Relative inhibition by specific soluble blood group substances of the isoagglutinins in the 0.02 M phosphate buffer fractions of 53 type O mothers

Blood type, newborn	No. of mothers tested	No. of isoagglutinins tested		Number of mothers with noninhibitable isoagglutinins Highest dilution of SSBGS which fails to inhibit $\frac{1}{500}$	$\frac{1}{100}$	$\frac{1}{40}$	$\frac{1}{20}$	$\frac{1}{10}$	$\frac{1}{1}$	$\frac{1}{4}$
A	13	α	11	7	3	1				
		β	5	2	3					
B	4	α	4	3				1*		
		β	4	3						1*
O	36	α	36	21	10		2	3†		
		β	17	2	7			5		3†
All	53	α	51	31	13	1	2	4	0	0
		β	26	7	10	0	0	5	0	4

* Same case; child had severe ABO erythroblastosis requiring transfusion.
† Only four sera represented; two contained both noninhibitable α and β, one contained only α and another only β.

mine that concentration which just failed to inhibit visible agglutination. These results, correlated with the ABO type of the newborn, are summarized in Table VI. The horse B substance used for these tests inhibited anti-B much less effectively than the hog A substance inhibited anti-A.

Five prenatal sera so studied for placenta-crossing antibodies were found to contain α and/or β agglutinins, neither of which could be neutralized by SSBGS at a concentration of 1 : 10 for A and 4 : 1 for B. Four of these patients delivered type O newborn, but one delivered a type B child which required exchange transfusion therapy for severe ABO erythroblastosis.

In 200 deliveries, approximately 90 type O mothers can be anticipated, and in the small series shown in Tables III, IV and V, 73 type O mothers were studied. Therefore, 0.8 case of ABO disease was expected (23), and one case was encountered. Type O mothers have incompatible offspring of types A_1 and B in approximately 30 per cent of instances (the sum of the gene frequencies of A^1 and B), so that for every case of erythroblastosis, an additional two to three unselected type O women should have similar ABO isoagglutinins which are of no importance to newborn of types O and A_2 (23). In the present series, four such women were encountered. One of these patients happened to be primagravida, consistent with the fact that ABO disease may be observed in the first incompatible pregnancy.

Table VI also shows that incompatible infants without evidence of hemolytic disease were delivered from 17 type O mothers who had demonstrable isoagglutinins in their 0.02 M fractions. In 15 instances these antibodies had the specificity of the A or B antigen found on the cord erythrocytes, and the titration values ranged from 1 : 4 to 1 : 160. The mothers of 10 incompatible newborn were found to have such incompatible isoagglutinins with a titer of at least 1 : 20. However, only one infant of this entire group had clinical disease, and the isoagglutinins in the 0.02 M fraction of this particular mother were highly resistant to inhibition by SSBGS, whereas the other nine were easily neutralized. Thus the inhibitable isoagglutinins in 0.02 M fractions, regardless of titer, did not appear to cause erythroblastosis, whereas the noninhibitable isoagglutinins so separated, even of low titer, furnished the expected correlation.

5. The nature of the isoagglutinins in the sera of mothers who have had infants with severe ABO erythroblastosis. Isoagglutinins eluted in 0.02 M fractions and resistant to inhibition by SSBGS are not common (Table VI), having been observed in only 5 of 110 prenatal specimens of blood types O, A and B. However, if such isoagglutinins are responsible for ABO erythroblastosis, they must occur in the sera of all women who have a history of children with severe clinical disease. Seven patients with typical histories, including the case

TABLE VII

Inhibition by hog (A) and horse (B) specific soluble blood group substances of the 0.02 M phosphate buffer fractions of maternal α and β isoagglutinins when there is a history of severe ABO erythroblastosis *

ABO type of affected child	Case no.	Titer (reciprocal) of 0.02 M fraction in the presence of SSBGS: α agglutinins, Amount of A substance added: 0†	1/500	1/100	1/40	1/20	1/10	β agglutinins, Amount of B substance added: 0†	1/500	1/100	1/40	1/20	1/10	4/1	Titer of native serum: α	β
A	21	160	nd‡	40	40	20	4	40	nd	40	40	20	20	0	1,280	320
A	22	80	20	20	nd	nd	4	2	0	0	nd	nd	0	0	160	20
A	42	160	160	80	nd	nd	20	4	2	2	nd	nd	0	0	640	160
A	64	80	80	20	nd	nd	6	20	20	6	nd	nd	2	0	2,560	160
A	94	160	nd	80	20	4	4	2	0	0	0	0	0	0	2,560	640
A	184	160	nd	20	10	4	2	160	nd	80	80	40	20	0	1,280	2,560
B	141	40	20	10	nd	nd	4	40	40	20	nd	nd	4	2	320	320

* All mothers, type O.
† No SSBGS added; titer of isoagglutinins.
‡ Not done.

from Table VI, were recalled for study, and the results are shown in Table VII.

The sera of all of these women with a history of severe ABO erythroblastosis in previous pregnancies revealed type-specific "noninhibitable" ABO isoagglutinins in a titer of at least 1:2 in their 0.02 M fractions. All newborn in this series required exchange transfusion treatment or developed kernicterus because treatment was not available. One mother (no. 42) had delivered her last child eight years previously, thereby revealing how persistent these antibodies may be.

6. The nature of the isoagglutinins in the serum of unselected type O donors immunized with SSBGS. Increasing severity of ABO erythroblastosis in later deliveries is seen occasionally. Significant increase in maternal ABO isoagglutinins has not been observed during the course of an incompatible gestation nor after the delivery of a nonsecretor infant (6). On the other hand, titer increases have been noted post partum following the delivery of some ABO incompatible secretors (6), presumably due to the SSBGS content of amniotic fluid. This raises the question as to whether SSBGS can incite noninhibitable isoagglutinins that can be eluted in 0.02 M fractions.

For information on this problem, the sera of five unselected type O donors, who had been immunized with both A and B SSBGS for preparation of anti-AB reagents (high titer type O sera), were fractionated on DEAE cellulose columns. The SSBGS which had been used for the immunization of these donors were of the same source and manufacture as those employed in the

TABLE VIII

Inhibition by hog (A) and horse (B) specific soluble blood group substances of the 0.02 M phosphate buffer fractions of the α and β isoagglutinins of unselected donors previously immunized with the same SSBGS

Case no.	Titer (reciprocal) of 0.02 M fraction in the presence of SSBGS: α agglutinins, Amount of A substance added: 0*	1/500	1/100	1/30	1/20	1/10	β agglutinins, Amount of B substance added: 0*	1/500	1/100	1/40	1/20	1/10	4/1	Titer of native serum: α	β
250	40	nd†	16	12	8	4	80	nd	80	80	80	40	10	2,048	2,048
251	40	nd	20	16	16	10	80	nd	30	30	30	20	10	1,024	1,024
253	60	nd	20	20	10	10	80	nd	80	40	40	10	8	512	2,048
254	>160	nd	160	40	20	20	>160	nd	>160	>160	<160	>160	8	2,048	2,048
255	>160	nd	30	20	20	4	>160	nd	>150	80	80	30	8	>2,408	1,024

* No SSBGS added; titer of isoagglutinins.
† Not done.

present inhibition studies. The 0.02 M fractions of all five donor sera were found to contain non-inhibitable α and β isoagglutinins, resembling those in the sera of the mothers of erythroblastic infants. The results are summarized in Table VIII.

DISCUSSION

Severe ABO erythroblastosis is familial and often observed in the first ABO incompatible child (5, 6). Although almost all subsequent incompatible infants are also affected, increasing severity of the disease is not seen regularly (12, 14). Thus ABO disease resembles Rh erythroblastosis in its familial incidence, but differs by not reflecting so much evidence of specific maternal isoimmunization by the products of conception.

Schiff (12) postulated that ABO erythroblastosis was to be expected when the maternal serum contained a special ("Type VI") pattern of antibodies highly resistant to neutralization by SSBGS; but, to explain the absence of hemolytic disease in the incompatible children of some type O mothers of this description, he proposed protection against the cross-reacting antibody described by Rosenfield and Ohno (7). Since Schiff studied whole maternal serum, his conclusions must be reviewed in the light of the properties of 7S maternal isoagglutinins.

If maternal isoagglutinins are to be responsible for erythroblastosis, they must not only cross the placental barrier but combine with fetal erythrocytes. In the case of ABO isoagglutinins, extra-erythrocytic antigens occur in many tissues independent of the ABO secretor status (15) and exert a protective effect of considerable magnitude, as evidenced by the low frequency of erythroblastosis despite the common occurrence of diffusible isoagglutinins (6, 8).

At least four components, with sedimentation constants of 7S, 19S, 28S, and 44S, have been found in the γ-globulin fractions of normal human serum (24), and recently Fahey and Morrison (20) showed that all of the ABO isoagglutinins have a sedimentation value of either 7S or 19S. The "non-γ-globulin" isoagglutinins reported by Abelson and Rawson (19) appear to have been due to conditions of the separation (20). Abelson and Rawson also reported that noninhibitable ABO isoagglutinins were almost all located in 7S fractions; but in all cases, and at the same concentration of SSBGS that they employed, we found only a small part of the total isoagglutinins to be noninhibitable and to occur in both the 0.02 M and the 1 M fractions if isoagglutinins were present at all in the former.

Table III reveals that most ABO isoagglutinins are not γ_2-globulins and do not cross the placenta. Poorly inhibitable isoagglutinins eluted in 1 M fractions, although not shown in this report, were observed frequently and would contribute misleading information.

Placental permeability and resistance to inhibition by SSBGS are certainly not the sole parameters of the problem of ABO erythroblastosis, but their contribution must be of primary consideration. Other contributing factors include the expression of erythrocytic ABO antigens because A_2 children tend to be spared (6); the secretor status because erythroblastotic infants tend to be secretors (5, 13, 6, 14); and the ability of the affected newborn to cope with a hemolytic syndrome (4). These and, probably, other variables must be studied. If the noninhibitable isoagglutinins of 0.02 M fractions are responsible for ABO erythroblastosis, there is now an opportunity to attack these secondary problems.

The present report raises an immediate problem concerning the nature of noninhibitable isoagglutinins shown to be associated with erythroblastosis. If these antibodies can be incited by SSBGS (Table VIII), they should not represent antigenic structural grouping differences between erythrocytes and SSBGS. Instead, these differences could reflect the heterogeneity of antibody molecules with respect to the size of their specific combining sites (25, 26). Kabat (25, 26) has demonstrated that antidextran molecules vary considerably in the size of their combining sites, with the upper limit being sites complementary to terminal nonreducing chains of six or seven glucose units in α-1,6 linkage. Thus, although erythrocytes of types A_1 and B might agglutinate with antibody molecules having combining sites of any size, SSBGS might form reversible complexes with some antibody molecules which could be released in sufficient amount to support visible agglutination under selected circumstances. Alternatively, nonspecific anamnestic response is required to explain the observations shown in Table VIII.

The term "immune" for agglutinins which cross the placenta, as opposed to "natural" saline agglutinins (8, 27), is not supported by the present data. All ABO agglutinins appear to be immune responses to antigenic structural groupings resembling those defined by Kabat (10) for human blood group substances.

The nature of the distinction between A_1 and A_2 red cells is also subject to review. Anti-A_1 reagents of human, animal or plant origin are highly susceptible to inhibition by SSBGS, and precipitate with SSBGS in common with anti-A (28). The distinction made by these reagents thus appears to be on a quantitative basis: there is more A on A_1 cells than on A_2 cells. On the other hand, anti-A inhibited with SSBGS toward A_2 cells, may persist in agglutinating A_1 cells. The problem of the specificity of noninhibitable anti-A, as related to the restricted occurrence of erythroblastosis in type A_1 offspring, is now under investigation.

The column chromatographic isolation of isoagglutinins which can cross the placenta appears to be a valuable method for the study of all forms of maternal isoimmunization in relation to erythroblastosis. This method is now being used for its applicability to Rh problems, and the results appear promising, but not identical with the report of Abelson and Rawson (29).

The column chromatographic method requires at least two days to perform and is not feasible for rapid diagnosis in the newborn period. For prenatal tests, the method appears to be practical.

SUMMARY AND CONCLUSION

1. ABO isoagglutinins associated with the bulk of 7S γ_2-globulins were separated from other serum proteins by anion exchange column chromatography.

2. The ABO isoagglutinin activity of 33 cord blood sera was found to be restricted to 7S γ_2-globulin-containing fractions.

3. Maternal serum ABO isoagglutinins were encountered chiefly in macroglobulin-containing fractions, but paired specimens of maternal and cord sera obtained at the time of delivery revealed that the 7S γ_2-globulins as well as the associated isoagglutinins existed in equilibrium on both sides of the placenta.

4. Out of 110 unselected prenatal sera, isoagglutinins, in a titer of at least 1:2 in acacia, were eluted in 0.02 M phosphate buffer, pH 6.3, in 53 of 73 type O mothers, but not in 28 type A and 9 type B women. Anti-A (α) was noted in 51 of these fractions, but anti-B (β) in only 27. These findings were considered to be consistent with the rarity of ABO erythroblastosis in offspring of mothers not of type O, and with the excess of erythroblastosis due to anti-A.

5. When these 53 examples of chromatographically separated isoagglutinins were tested for resistance to inhibition by specific soluble blood group substances (SSBGS) (hog A and horse B), only 5 contained markedly resistant antibodies. Four of these mothers delivered type O children, but one mother delivered a type B child who had erythroblastosis and required exchange transfusion therapy. An additional nine mothers in this series also delivered ABO-incompatible newborn, and their 0.02 M fractions contained type-specific isoagglutinins in a titer ranging from 1:20 to 1:160, but in each instance these antibodies were readily inhibitable with SSBGS, and clinical erythroblastosis was not observed.

6. Six other women with a history of having had one or more children with severe ABO erythroblastosis, and five unselected donors who had been immunized previously with SSBGS were all found to have such type-specific noninhibitable isoagglutinins.

7. The use of these methods in prenatal tests is implied.

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EFFECTS OF TRIPARANOL (MER–29) ON CHOLESTEROL BIOSYNTHESIS AND ON BLOOD STEROL LEVELS IN MAN *

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The possibility of reducing elevated blood cholesterol levels with agents that inhibit endogenous biosynthesis of cholesterol is an attractive one, since dietary treatment is sometimes difficult to achieve and almost always difficult to maintain. Some of the theoretical aspects of such an approach have been outlined (3, 4), and the feasibility of the approach has been established in experimental animals. For example, it has been shown that Δ^4-cholestenone, a potent inhibitor of cholesterol biosynthesis (5), can indeed lower levels of blood cholesterol (3, 6). Unfortunately, the feeding of Δ^4-cholestenone in man and animals leads to accumulation of dihydrocholesterol in the serum (7) and this latter compound is known to be itself atherogenic (8). Certain other inhibitory substances have been explored but either have proved to be relatively ineffective (9, 10) or have not yet been adequately tested clinically (11, 12).

In 1959 Blohm, MacKenzie, Kariya and Laughlin at the William S. Merrell Company reported their finding of a new inhibitor of cholesterol synthesis, MER-29 (1-[*p*-β-diethylaminoethoxy)-phenyl]-1-(*p*-tolyl)-2-(*p*-chlorophenyl) ethanol). Their studies indicated that the compound blocked synthesis at some point subsequent to the formation of lanosterol (13) and that serum and tissue levels of cholesterol were reduced in experimental animals (14). Animal studies in this laboratory demonstrated the accumulation of desmosterol (24-dehydrocholesterol) in the tissues of MER-29-treated animals, and a series of isotopic studies established that the major site of action of the drug was at the last step in cholesterol biosynthesis—namely, in the reduction of desmosterol to cholesterol (1, 15, 16). The present paper reports clinical studies which show that the mechanism of action in man is similar to that in animals. The degree of accumulation of desmosterol in the serum of treated patients and the changes in cholesterol level are reported. The analytical problems involved in determining serum desmosterol levels are discussed, and the feasibility of using gas-liquid chromatography for separation of desmosterol and cholesterol is demonstrated. Finally, the rates of esterification of serum desmosterol and serum cholesterol are compared.

METHODS

Patients were admitted to a metabolic ward of the Clinical Center and placed on an isocaloric diet of constant composition (three patients, studied in the outpatient department, followed a prescribed low animal fat diet, but its composition was not measured). The patients were maintained on their diets for a period of at least 2 weeks before treatment was begun. Then MER-29 was given orally in a single daily dose of either 100 or 250 mg.

Blood samples from the antecubital vein were taken in the morning, two or three times a week, after an overnight fast. The serum samples were analyzed for cholesterol and desmosterol as described below.

Determination of cholesterol and desmosterol in serum. Sera were extracted with 25 vol of 1:1 alcohol:acetone (vol/vol) and the free sterols were precipitated from aliquots of these extracts as digitonides. Other aliquots were treated with KOH for 1 hour at 40° C, neutralized with acetic acid and the total sterols were precipitated as digitonides. The sterol digitonides derived from the free and the total sterol fractions were washed with 50 per cent aqueous alcohol, 2:1 ether:acetone (vol/vol) and ether, and dried. The precipitates were cleaved with dry pyridine at 100° C for 10 to 30 minutes. Twenty vol of ether was then added, the precipitated digitonin was separated by centrifugation and the precipitate was washed once with ether. The pooled ether supernatants were taken to dryness and the sterols were re-extracted with dry ether, which was again removed by evaporation. The sterols were determined by the method of Abell, Levy, Brodie and Kendall (17) modified to permit separate estimation of cholesterol and desmosterol, as described in

* Preliminary reports of this work were presented at the Conference on MER-29 (Triparanol), December 1959, Princeton, N. J. (1) and at the meetings of the Federation of American Societies for Experimental Biology, April 1960, Chicago, Ill. (2).

detail elsewhere (18). This method depends on reading the optical densities of the products of the Liebermann-Burchard reaction at 635 mμ after 35 minutes and at 420 mμ after 90 minutes. Calculation of the amounts of cholesterol and desmosterol present is based on the different absorption spectra of the colored products derived from the two sterols. Standards of pure cholesterol and desmosterol were analyzed with each series of determinations. Pure desmosterol was obtained, either by isolation from livers of rats fed MER-29 (18) or synthetically from 25-norketocholesterol by methylation with a Grignard reagent and dehydration of the resulting 25-hydroxycholesterol [1] (19).

Radioisotopic studies. On the mornings when radioisotopic studies were initiated, food was withheld until after the collection of the 4-hour blood sample. Twenty-five μc of a saline solution of 2-C^{14}-mevalonic acid (approximately 8 mg) was rapidly injected into the antecubital vein. Blood samples were taken at 1, 2, 3, 4, 12, and 24 hours, the first samples from the opposite arm. In one study samples were also taken at 7, 14, and 21 days.

The free sterols were precipitated as digitonides from extracts of 10 to 50 ml of serum and the precipitate was washed as described for the analytical determination. The combined supernatant and washes were evaporated to dryness and the residue was saponified in ethanolic KOH. The nonsaponifiable materials were extracted with heptane and the extract was backwashed with water. The heptane extract was taken to dryness, the residue was redissolved in ethanol and the sterols were precipitated with digitonin and washed as usual. The digitonides of both the free and the esterified sterol fractions were subsequently cleaved with pyridine and extracted with ether as described above. Small aliquots of these samples were taken for counting of radioactivity and for colorimetric determination, which permitted the calculation of the specific radioactivities of the unfractionated free and esterified sterols. In some studies the cholesterol and desmosterol in both free and ester sterol fractions were separated by chromatography of their yellow-colored *p*-phenylazobenzoyl esters on columns of silicic acid : Celite 2 : 1 (20). Some of the samples to be chromatographed contained only 5 to 10 mg of total sterols, and small columns, 8 mm in diameter, had to be used. On this small scale complete separation of the two sterol bands was not obtained. After the yellow ester zone had run almost to the bottom of the column, the silicic acid cylinder was mechanically extruded and slices were taken from the slowest and fastest moving portions. These zones were individually extracted with benzene. After saponification in methanolic KOH, the sterols were extracted with heptane, washed with water and divided into two aliquots for counting of radioactivity and for colorimetric determination of cholesterol and desmosterol. Knowing the analytical values for the two sterols in the samples counted, it was possible to calcu-

[1] The authors wish to thank Dr. E. Mosettig and Mr. R. T. Brown for preparing the synthetic material.

TABLE 1

Serum concentrations of desmosterol reached during treatment with MER-29 and comparison of free/total ratios for cholesterol and for desmosterol

Patient	MER-29 dosage	Days on drug	Total sterols		Total sterols as desmosterol	Free sterols		Free sterols as desmosterol	Free/total ratio	
			Cholesterol	Desmosterol		Cholesterol	Desmosterol		Cholesterol	Desmosterol
	mg/day	no.	mg %		%	mg %		%		
A.T.M.	100	15	219	55	20	61	14	19	0.28	0.25
M.V.B. (1)*	100	22	205	155	43	60	39	39	0.29	0.25
M.V.B. (2)*	100	21	191	114	37	53	31	37	0.28	0.27
M.S.C.	100	22	105	29	22	32	7	18	0.30	0.24
L.J.McC.	100	29	153	43	22	48	9	16	0.31	0.21
G.B.	100	59	164	48	23					
W.F.B.	100	67	104	25	19	37	10	27	0.35	0.40
E.H.	250	14	151	50	25	46	12	21	0.30	0.24
J.L.C.	250	17	143	50	26	48	20	42	0.33	0.40
P.J.B.	250	101	89	39	30	28	15	54	0.31	0.38
					Mean 26.7			Mean 30.3	Mean 0.31	Mean 0.29

* Two trials of the drug, separated by about 6 weeks.

late the two specific radioactivities individually, even though the desmosterol was contaminated with a small amount of cholesterol and vice versa.

Separation of sterols by gas-liquid chromatography was done according to vanden Heuvel, Sweeley and Horning (21). The column was packed with Chromosorb W, coated with silicone rubber (G.E. SE-30). The chromatographic runs were carried out at 220° C.

RESULTS

As shown in Table I, all patients treated with MER-29 had desmosterol in their serum. On the other hand, attempts to demonstrate desmosterol in normal serum, both by column chromatography of *p*-phenylazobenzoyl esters of the total sterol fraction and directly by gas-liquid chromatography, were unsuccessful. The sensitivity of these methods as applied is such that the level of desmosterol in normal serum can be no greater than 0.1 per cent of the total sterols. During MER-29 treatment, in most patients the serum desmosterol reached a level of between 25 and 55 mg per 100 ml, or on the average, about 27 per cent of the total circulating sterols. Patient M.V.B. showed quite a distinctive response, reaching desmosterol levels of 155 and 114 mg per 100 ml, respectively, in two separate trials of the drug at 100 mg per day. In this case the desmosterol in the serum represented about 40 per cent of the total circulating sterols.

The time course of the appearance of desmosterol in the serum is typified by the results shown in Figure 1. The desmosterol concentration rose rather quickly after the start of treatment, reached a plateau in about 2 weeks and then remained flat during continued treatment. Within 2 weeks after discontinuing the drug, desmosterol had disappeared from the serum. There was no evidence for continuing accumulation of desmosterol in the serum, and after a period of drug treatment it reached a fairly constant level characteristic for each patient. A similar time course in two separate trials of the drug in Patient M.V.B. is shown in Figure 2.

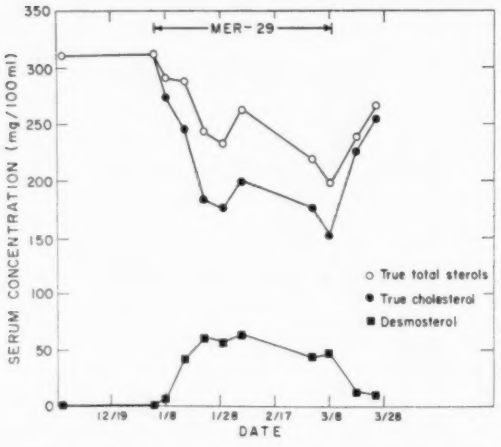

FIG. 1. TIME COURSE OF SERUM CHOLESTEROL AND DESMOSTEROL CONCENTRATIONS IN PATIENT G.B. RECEIVING MER-29.

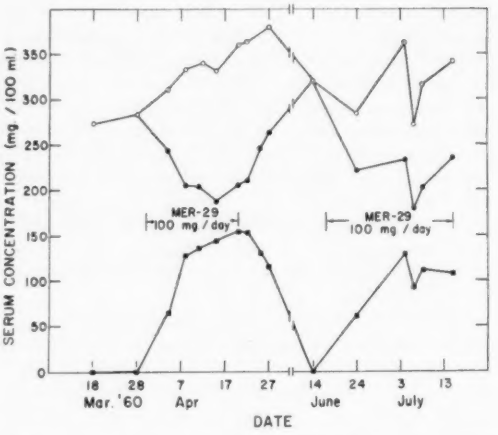

FIG. 2. TIME COURSE OF SERUM CHOLESTEROL AND DESMOSTEROL CONCENTRATIONS IN PATIENT M.V.B. RECEIVING MER-29 IN TWO SEPARATE TRIALS. Symbols the same as those in Figure 1.

Although there was some variability, the relative amounts of desmosterol present in the free sterol fraction and the relative amounts of desmosterol in the total sterol fraction (averages 30.3 and 26.7 per cent, respectively) were comparable. As can be seen in Table I, the free to total ratio for circulating desmosterol was not significantly different from the free to total ratio for circulating cholesterol.

Evaluation of changes in serum sterol levels. As has been previously reported (1), the color yield of desmosterol in the Liebermann-Burchard reaction is only about 60 per cent of the color yield given by cholesterol. Consequently, use of this method, the basis of most clinical procedures for serum sterol analysis, will give misleading results in patients under treatment with MER-29. In the present studies, use was made of the differen-

tial colorimetric method of Avigan and co-workers (18) to determine the true levels of cholesterol and desmosterol.

Preliminary results using gas-liquid chromatography (21) suggest the usefulness of this technique, when available, for estimating desmosterol in the serum.[2] An analytical record obtained of the total digitonin-precipitable sterol fraction of a patient under treatment with MER-29 is shown in Figure 3. Cholesterol emerged first with a retention time of 36 minutes and desmosterol followed, with a retention of 39.7 minutes. Because of an overlap and some tailing, precise measurement of relative concentrations was not possible, but the calculated results agreed well with those obtained on the same sample using the differential colorimetric method. Different liquid phases and different conditions for chromatography are under study in attempts to improve this resolution. It is of interest to note that at the sensitivity employed no sterols other than desmosterol and cholesterol were detected in the digitonin-precipitable fraction of this patient's serum.

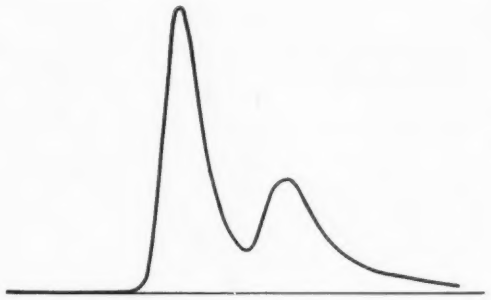

FIG. 3. GAS CHROMATOGRAM OF STEROLS ISOLATED FROM SERUM OF PATIENT M.V.B. TREATED WITH MER-29. The larger peak, cholesterol; the smaller, desmosterol.

The true cholesterol levels in patients during control periods and under treatment with MER-29 are summarized in Table II. The nature of the diet is indicated in the last column of the table. Every patient showed a marked drop in true cholesterol level; the absolute changes ranged from 39 to 131 mg per 100 ml (average 83); the percentage changes ranged from 18.9 to 46.3 per cent (mean 34.0). It should be noted that neither the

[2] We acknowledge with thanks the helpful assistance of Dr. W. J. A. vanden Heuvel, Dr. E. C. Horning and Dr. A. Karmen in these gas-liquid chromatographic studies.

TABLE II

*Changes in true * total serum sterol levels and changes in apparent † (Liebermann-Burchard) serum sterol levels with MER-29 treatment*

Patient	Dosage of MER-29	Days on drug	Control cholesterol level	True cholesterol level on therapy	True total sterol level on therapy	Change in true sterols	*Apparent* sterol level on therapy	Change in apparent sterols	Diet
	mg/day	*no.*	*mg %*	*mg %*	*mg %*	*%*	*mg %*	*%*	
L.J.McC.	100	27	219	152	192	−12	178	−19	35% calories, animal fat
J.L.C.	250	16	206	167	207	0	191	− 7	
M.J.C.	100	19	174	112	141	−19	130	−25	
W.F.B.‡ (1)	100	20	264	172	206	−22	192	−27	20% calories, animal fat
W.F.B. (2)	100	50	233	148	190	−18	174	−25	
G.B.§	100	21	311	180	239	−23	216	−30	Low animal fat
A.T.M.§	100	15	289	219	274	− 5	252	−13	
P.J.B.§	250	109	166	89	118	−29	102	−39	
E.H.	250	14	227	148	192	−15	175	−23	40% calories, corn oil
						Mean −16		Mean −23	
M.V.B.‡ (1)	100	17	302	187	332	+10	274	− 9	60 % calories, corn oil
M.V.B. (2)	100	21	310	211	233	+ 7	288	− 7	

* Determined by the differential colorimetric method described in the text.
† Value that would have been obtained based on color yield of cholesterol alone.
‡ These patients had two independent trials on the drug separated by several months.
§ Outpatients.

TABLE III

Comparison of specific radioactivities of serum free desmosterol and serum free cholesterol 4 hours after intravenous injection of 2-C^{14}-mevalonic acid in patients under treatment with MER-29

Experiment no.	Specific radioactivity	
	Free desmosterol	Free cholesterol
	cpm/mg	
1	1,765	11
2	1,949	0
3	838	62

absolute nor the relative changes in cholesterol level were distinctly different in the patients consuming animal fat (containing from 600 to 1,000 mg of cholesterol daily) from the changes seen in patients on corn oil formula (containing virtually no cholesterol).

Because of the simultaneous accumulation of desmosterol in the serum, the changes in true total serum sterol level were much less striking. Six patients showed a drop, in one there was no change, and in one there was actually a rise (Table II). The results of individual time course studies in Patient W.F.B., whose response was fairly typical, and in Patient M.V.B., whose total sterol level rose under treatment, are shown in Figures 1 and 2, respectively. If the results in Patient M.V.B. are excluded and the results of the two separate trials in Patient W.F.B. are averaged, the mean drop in true total serum sterol level was 15 per cent. If all the results are grouped together the mean drop was 11 per cent.

The calculated changes in *apparent* sterol level that would have been obtained using standard clinical methods based on the Liebermann-Burchard color reaction are also shown in Table II. Because of the lower color yield given by desmosterol, the *apparent* changes are greater, averaging 23 per cent if Patient M.V.B. is excluded or 21 per cent for the group as a whole.

It is pertinent to note that there was a drop in apparent sterol level in the case of Patient M.V.B. even though the true sterol level actually rose slightly.

Radioisotopic studies. In patients under treatment with MER-29, the specific radioactivity of serum free desmosterol 4 hours after intravenous injection of 2-C^{14}-mevalonic acid was at least an order of magnitude higher than the specific radioactivity of serum free cholesterol (Table III), compatible with a precursor product relationship. The data shown in Figure 4, from a study in which the specific radioactivities of the two sterols were followed for 3 weeks after injection of labeled mevalonate, also support the conclusion that desmosterol is an immediate percursor of cholesterol. The specific radioactivity of desmosterol remained higher than that of cholesterol for about 2 weeks, at which point the specific radioactivity curves intersected. These results support the interpretation that in man, as in experimental animals, the drug effectively blocks the reduction of desmosterol to cholesterol. That the inhibition of this reaction is quite marked is shown by the length of time taken for the curves of specific radioactivity to cross.

As shown in Figure 4, the curve of specific radioactivity of ester desmosterol rose during the first 48 hours after injection of 2-C^{14}-mevalonate, crossed the curve for free desmosterol and then fell in parallel with, but slightly above it. The relation between the curves is that of precursor and

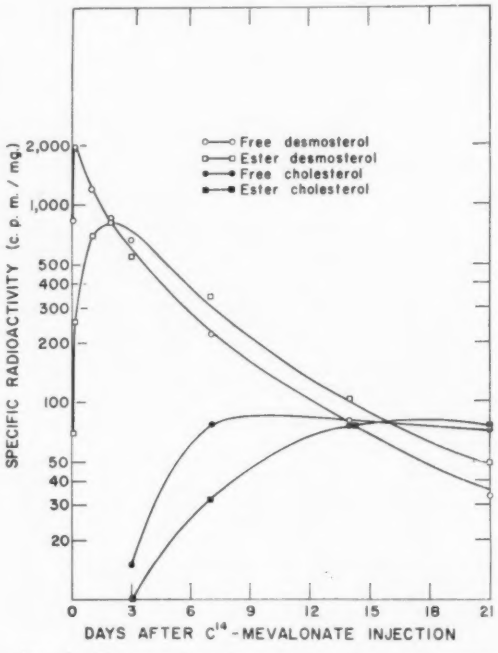

FIG. 4. SPECIFIC RADIOACTIVITY OF FREE AND ESTERIFIED DESMOSTEROL AND CHOLESTEROL IN SERUM OF PATIENT W.F.B. FOLLOWING THE ADMINISTRATION OF C^{14}-MEVALONIC ACID.

product (22). The rate of conversion of free desmosterol to ester desmosterol appears to be quite similar to that reported for cholesterol by Hellman (23) and Eckles (24), and their coworkers and is confirmed in the control studies reported below.

A series of experiments was carried out, in which the rate of appearance of radioactivity from injected 2-C^{14}-mevalonate was determined during drug treatment and during a control period. Each patient served as his own control in these studies. The specific radioactivity of the unfractionated digitonin-precipitable sterols was determined in the hope of establishing whether or not the overall rate of synthesis and delivery of the sum of the two sterols into the serum was reduced by drug treatment.

Representative results are shown in Figures 5 and 6. In Figure 6 there was no lowering effect of MER-29 on the incorporation of label into serum sterols. The general shape of the curves is little affected by treatment with MER-29. As can be seen from the results in Table III, most of

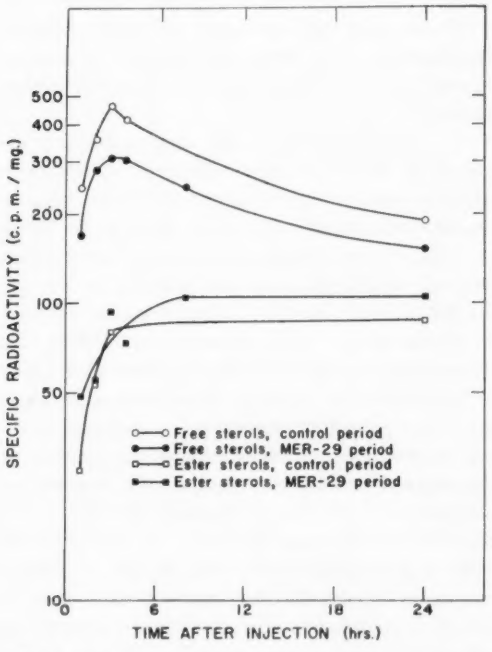

FIG. 5. SPECIFIC RADIOACTIVITY OF FREE AND ESTERIFIED UNFRACTIONATED STEROLS IN SERUM OF PATIENT M.J.C. DURING CONTROL AND MER-29 PERIODS.

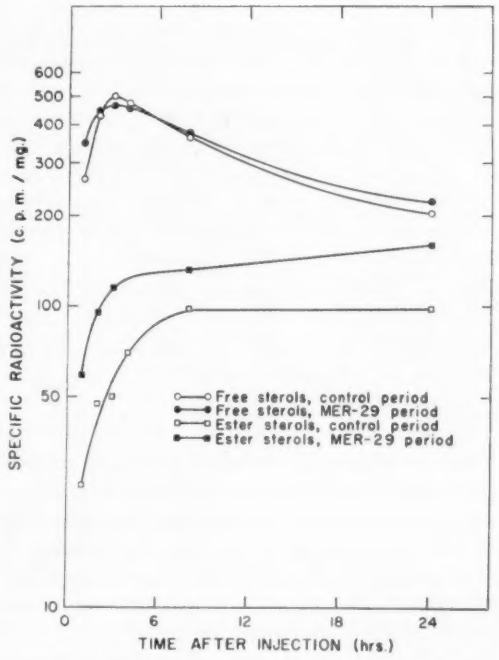

FIG. 6. SPECIFIC RADIOACTIVITY OF FREE AND ESTERIFIED UNFRACTIONATED STEROLS IN SERUM OF PATIENT L.J.McC. DURING CONTROL AND MER-29 PERIODS.

the radioactivity in the serum sterols during the first few hours of each experiment during drug treatment was present as desmosterol. The fact that the specific radioactivtiy of the ester sterol approaches the specific radioactivity of the free sterols at similar rates during experimental and control periods indicates, then, that the esterification of desmosterol proceeds at a rate comparable with that for esterification of cholesterol in the normal individual. This is consistent with the analyses for free to ester ratio discussed above, which indicate that the steady state degree of esterification of desmosterol is comparable with that of cholesterol.

Comparison of the specific radioactivities of serum free sterols 3 hours after injection of radioactive mevalonate, during a control period on the one hand and during MER-29 treatment on the other hand, shows a quantitatively variable but qualitatively consistent difference. On the average, the peak specific radioactivity during treatment was 16 per cent lower than that during the control period (Table IV). During the period of

TABLE IV

Specific radioactivity and total radioactivity of unfractionated serum sterols 3 hours after 2-C^{14}-mevalonate injection during a control period and during treatment with MER-29

Patient	*Specific* radioactivity of unfractionated serum free sterols 3 hrs after injection of 2-C^{14}-mevalonate			*Total* radioactivity (unfractionated serum free sterols/100 ml serum) 3 hrs after injection of 2-C^{14}-mevalonate		
	Control period	MER-29 period	Difference	Control period	MER-29 period	Difference
	cpm/mg		%	*cpm/100 ml*		%
P.J.B.	501	448	−11	24,000	17,900	−25
W.F.B.	616	545	−12	38,200	32,700	−14
M.V.B.	650	660	+ 2	57,200	50,800	−11
M.J.C.	461	308	−33	22,100	12,300	−44
L.J.McC.	501	468	− 7	31,500	25,700	−18
G.B.	411	254	−38			
			Mean −16.5			Mean −22.4

MER-29 treatment, however, the absolute concentration of free sterols was lower than during the control period. Consequently, the difference in *total* radioactivity in the serum free sterols is even greater. Analyzed in this way, the synthesis and delivery of radioactive sterol to the serum was 22 per cent less during treatment.

DISCUSSION

Treatment with MER-29 in this limited series of cases led consistently to a marked decrease in the true cholesterol concentration in the serum (mean depression, 34 per cent). There was a simultaneous appearance of desmosterol in the serum at concentrations generally ranging from 25 to 55 mg per 100 ml. In a patient with familial hypercholesterolemia and xanthoma tendinosum, the desmosterol concentration in the serum rose to well over 100 mg per 100 ml. In this case the total serum sterol level actually rose slightly under treatment. If this case is excluded from the results, the mean drop in true sterol level was 16 per cent (range 0 to 23). These results cannot be directly compared with those of most of the clinical trials reported in the literature, because the investigators, not yet being aware of the occurrence of desmosterol in the serum and the analytical problem posed by it, have generally used simpler methods based on the Liebermann-Burchard reaction (25–27). Because of the low color yield given by desmosterol, use of such methods gives a result that is too low for the true serum sterol level. In the present series, for example, the change in serum sterol level as a result of treatment, estimated by use of the Liebermann-Burchard reaction, would have been 23 per cent rather than the true change of 16 per cent. Recently, Hollander, Chobanian and Wilkins (28) reported results in a series of patients in whom the true total serum sterol level was determined by measuring total digitonin-precipitable sterols gravimetrically. In 12 patients treated for 2 to 47 weeks they found a mean drop in true sterol level of 17 per cent and estimated desmosterol levels ranging from 8 to 83 mg per 100 ml. These results and those of the present study are in rather good agreement.

Any interpretation of the therapeutic significance of the study depends importantly upon whether desmosterol is considered to be equipotential with cholesterol as an atherogenic agent or whether its atherogenicity is different from that of cholesterol. As yet there is no experimental basis for deciding objectively on an answer to this problem. Still, it is worthy of note that desmosterol is remarkably like cholesterol in many of its properties. Its physical properties are similar to the point that separation by ordinary methods is extremely difficult. The distribution of desmosterol among the lipoprotein fractions is comparable with that of cholesterol (29). The rate of esterification of desmosterol, as shown here, is quite comparable with the rate of esterification of cholesterol in the same individual and the steady state free/ester ratio for desmosterol is the same as that for cholesterol. Animal studies have shown that, with high dosages of MER-29, desmosterol represents a major sterol component

in many body tissues (18), indicating that it can replace cholesterol in the structural components of tissues, including red blood cells. All of these points taken together make it seem unlikely that the rate of deposition of desmosterol into atheromata will be found to be significantly different from that of cholesterol. At least until there is evidence to the contrary, the most reasonable hypothesis is that the two sterols are equipotential in atherogenicity.

Should this be the case, then the results of treatment must be evaluated on the basis of changes in *total* sterol level. These changes are variable from patient to patient, and it is important to recognize that some patients show no response or even an increase in total sterols. On the other hand, there are some patients in whom the total sterol level drops significantly, and the mean drop is in the neighborhood of 15 per cent. This degree of response compares unfavorably with the therapeutic responses that have been obtained using dietary treatment with unsaturated fat, or low fat diets, or with nicotinic acid (30–32).

In view of what appears to be a very marked inhibition of endogenous cholesterol production, it might have been anticipated that responses of cholesterol level would be exaggerated in the absence of exogenous cholesterol. Although only a few patients are available for comparison, it is of interest to note that there appeared to be no strikingly different response in patients on a diet devoid of cholesterol than in the patients receiving exogenous cholesterol (1).

The results of the present study lead us to conclude that the mechanism of action of MER-29 in man is, as in experimental animals, an inhibition of the reduction of desmosterol to cholesterol. The conclusion is supported by the accumulation of desmosterol in the serum and in the red blood cells during treatment. It is further supported by the isotopic data showing that, after injection of 2-C^{14}-mevalonic acid, the specific radioactivity of desmosterol in the serum is one to two orders of magnitude higher than that of cholesterol in the serum. These specific radioactivities did not approach each other until about 2 weeks after injection of the labeled precursor, indicating that the degree of inhibition is marked.

The studies, in which the rate of appearance of radioactivity in unfractionated serum sterols (desmosterol plus cholesterol) was measured, are difficult to interepret. The peak specific radioactivity during treatment with MER-29 was slightly lower (mean 16.5 per cent). The results when expressed in terms of the total radioactivity in circulating sterols showed a somewhat more impressive difference (mean 24.4 per cent). Whichever way the results are expressed, there does appear to be an inhibition in the rate of synthesis of labeled sterols and their delivery into the serum. However, the percentage inhibition is relatively small despite the fact that the block in conversion of desmosterol to cholesterol appears to be quite effective, as shown by the slowness with which the specific radioactivity of cholesterol rises (Figure 4). One tentative interpretation must be that, although there is a significant degree of inhibition of the conversion of desmosterol to cholesterol, leading to a reduction in serum cholesterol levels, the decreased rate of delivery of cholesterol into the serum is virtually compensated by the increased rate of delivery of desmosterol. The interpretation is complicated by the facts that the pool of desmosterol is smaller than the pool of cholesterol and that the exchange of each of the two labeled sterols between tissue compartments may be proceeding at a quite different rate. Of particular relevance is the rate at which labeled sterol, newly synthesized in the liver, gets into the serum by isotopic exchange reactions relative to the rate at which it enters the serum compartment as a result of net secretion of sterol-containing lipoprotein molecules.

MER-29 is the first pharmacological inhibitor of cholesterol biosynthesis for which a definite site of action has been established. The drug appears to be free of at least short-term undesirable side effects, and it depresses total serum sterol concentrations. However, because of the accumulation of desmosterol, the response in terms of total sterol levels is not dramatic. Moreover, the long-term biological effects of desmosterol remain to be evaluated.

SUMMARY

1. In the nine cases studied, treatment with MER-29 was always associated with the appearance of desmosterol in the serum, which accounted for 19 to 43 per cent (average 27) of the total circulating sterols. The free to total ratio for

circulating desmosterol was not significantly different from that of cholesterol.

2. All of the patients treated showed a marked drop in serum cholesterol concentration (average 34.0 per cent) but because of the simultaneous accumulation of desmosterol, total sterol levels were depressed by only 15 per cent.

3. A method for determining desmosterol in the presence of cholesterol is described. It is demonstrated that the usual analytical procedures yield low values for total sterol levels when desmosterol is present.

4. After intravenous injection of 2-C^{14}-mevalonate in patients under treatment with MER-29, the specific radioactivity of serum desmosterol was at least an order of magnitude higher than that of cholesterol. The specific radioactivities of the two sterols did not become equal until 2 weeks after injection of labeled mevalonate.

5. The specific radioactivity of esterified desmosterol, low during the early hours, rose and became equal to the specific radioactivity of free desmosterol at 24 to 48 hours after injection of labeled mevalonate.

6. After injection of 2-C^{14}-mevalonate, the peak specific radioactivity and total radioactivity in the unfractionated serum sterols during treatment with MER-29 were significantly lower than during control periods.

7. It is concluded that the mechanism of action of MER-29 in man is to inhibit the reduction of desmosterol to cholesterol and that the depression of serum cholesterol levels and total serum sterol levels is attributable to this inhibition at the final step in cholesterol biosynthesis.

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THE SERUM LIPIDS IN MEN RECEIVING HIGH CHOLESTEROL AND CHOLESTEROL-FREE DIETS *

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In a previous study we found that dietary cholesterol provided in the form of egg yolk had a great effect upon the serum cholesterol and phospholipid levels in healthy human subjects (2). This effect was obtained with the use of high cholesterol and cholesterol-free diets which were balanced closely in the amounts of fat and in fatty acid composition. The range of cholesterol intake so tested was from 1,700 to 4,800 mg per day, amounts clearly above the usual daily American consumption of 500 to 1,000 mg. The present investigation was designed to examine the serum lipid responses of six men given dietary cholesterol as egg yolk in amounts compatible with usual dietary intakes. The effects of egg yolk cholesterol and crystalline cholesterol were also compared.

METHODS

Subjects. Six men, aged 41 to 52 years, were selected from prison volunteers and hospitalized on a metabolic ward for 13 weeks. After a complete medical examination they were found to be in good health; none was obese. Their urinalyses, blood counts, erythrocyte sedimentation rates and chest X-rays were normal.

Dietary plan. Initially, the men received a general diet for 1 week (3,000 calories, 70 g protein, 120 g fat, and 425 mg cholesterol). They then were given various experimental formulas as the sole source of nourishment for the next 12 weeks. Each man received the formula in three separate meals administered through an intragastric tube in order to insure precise quantities of each nutrient.

Aside from differences in their content of egg yolk and crystalline cholesterol, the formulas of all subjects were identical for each period (Table I). The caloric content was 3,000; the protein was 70 g derived from casein, except during period II when small components of the total protein were derived from egg yolk. The carbohydrate was supplied from a mixture of cornstarch, Dextrimaltose and sucrose. The basic formula contained normal amounts of required vitamins and minerals.

In each period the fat supplied 40 per cent of the total calories. It consisted of a vegetable oil mixture, 60 per cent peanut oil, 30 per cent cocoa butter and 10 per cent safflower oil. It was designed to have a fatty acid composition similar to that of egg yolk. During period II small amounts of fat were provided from egg yolk—i.e., from 8 to 25 per cent of the total fat for the three different groups. The fatty acid composition of the egg yolk and vegetable oil mixtures was determined by gas-liquid chromatography.[1] The fatty acid data of Table I were computed on the basis of these analyses. Thus, the iodine values and the percentage of saturated, monoethenoid and polyethenoid fatty acids were virtually identical for all periods and all subjects. Only the quantity of cholesterol in the diet was varied.

The diets were changed every 3 weeks in order to compare the effects of cholesterol-free diets with diets containing cholesterol. These periods were numbered from I to IV chronologically. The diets of periods I and III were cholesterol-free and identical for all subjects. The diet of period II contained cholesterol in the form of egg yolk: the two men in group A received 475 mg per day, the two men in group B consumed 950 mg, and the two men in group C received 1,425 mg. During period IV, crystalline cholesterol was blended into the formulas in a different amount for each group: group A 1,200 mg per day, group B 2,400 mg, and group C 3,600 mg.

Laboratory studies. Blood was drawn twice weekly from each subject after a 14 hour fast. The total cholesterol (3, 4), phospholipid (5) and triglyceride (6) were determined in duplicate on each serum specimen during all periods. The two serum determinations for each subject during the last week of each dietary period were averaged to provide a final figure which then was used for an indication of the effect of a given diet.

The Zak method for serum cholesterol determination was found to have improved accuracy when performed at a constant room temperature of 23° to 24° C and when the concentrated sulfuric acid was added rapidly to the ferric chloride-glacial acetic acid solution containing the

* Presented in part at the Annual Meeting of the American Heart Association, Council on Arteriosclerosis, St. Louis, October 21, 1960 (1). Supported by research grants from the National Institutes of Health, the American and Iowa Heart Associations, the Nutrition Foundation, the National Vitamin Foundation, and the American Cancer Society.

[1] Through the courtesy of Dr. Fred Mattson, Procter & Gamble Co.

TABLE I

Compostion of the formulas for the different dietary periods *

Period	Subjects	Cholesterol	Source of fat: Egg yolk	Source of fat: Vegetable oil mixture†	Iodine value	Fatty acids: Saturated	Fatty acids: Monoethenoid	Fatty acids: Polyethenoid	Protein: Casein	Protein: Egg yolk	Cystine
		mg	*g*	*g*		%	%	%	*g*	*g*	*g*
I Cholesterol-free	All	0	0	133	86	33.5	40.4	27.6	70	0	0.3
II High cholesterol (egg yolk)	Group A 1, 2	475	11	122	85	33.7	40.9	26.8	64.5	5.5	0.3
	Group B 3, 4	950	22	111	84	33.9	41.2	25.8	58.9	11.1	0.5
	Group C 5, 6	1,425	33	100	83	34.0	41.9	25.3	53.4	16.6	0.6
III Cholesterol-free	All	0	0	133	86	33.5	40.4	27.6	70	0	0.3
IV High cholesterol (crystalline cholesterol)	Group A 1, 2	1,200									
	Group B 3, 4	2,400	0	133	86	33.5	40.4	27.6	70	0	0.3
	Group C 5, 6	3,600									

* All subjects received 3,000 calories for each dietary period. Carbohydrates: cornstarch 75 g; Dextrimaltose no. 1 (Mead Johnson) 100 g; sucrose 207 g. Vitamins and minerals were added to meet the daily recommended allowance of the National Research Council and maintained constant through all periods. During period II, 34, 68, and 102 g of egg yolk were given to groups A, B, and C, respectively.
† Vegetable oil mixture: 60% peanut oil, 30% cocoa butter and 10% safflower oil.

extracted serum and immediately mixed with a stirring rod having a footplate. Although the Zak method for serum cholesterol provides results comparable with other methods (3), an analysis of each serum sample for total cholesterol was performed also in duplicate by the method of Abell, Levy, Brodie and Kendall (4). In all instances the results obtained for each of these healthy subjects under the different dietary conditions were comparable with the Zak method. The values reported are those determined by the Zak method.

For periods III and IV the total stool fat was determined with the Goldfish apparatus on 3-day collections.

The egg yolks fed in the experimental diet were pooled and an aliquot saved for chemical analysis. Cholesterol

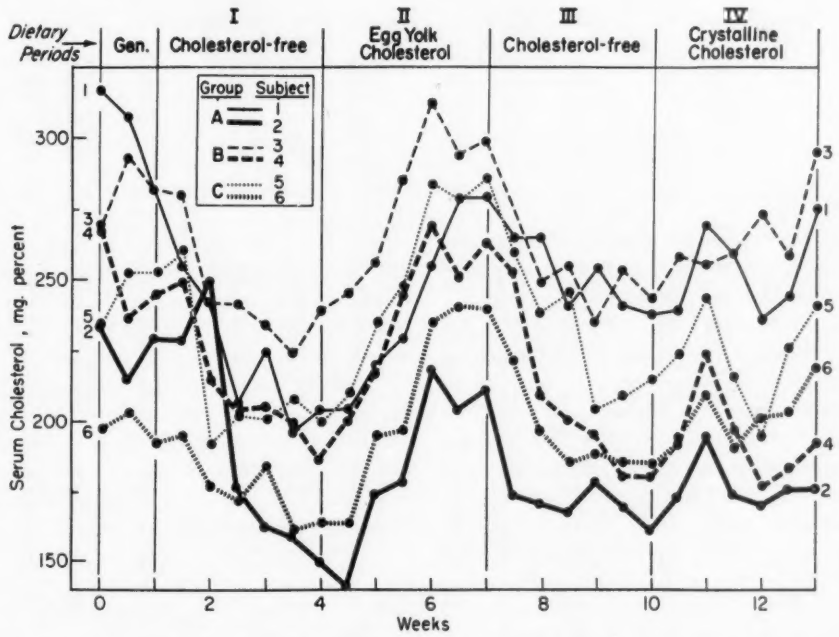

FIG. 1. THE SERUM CHOLESTEROL VALUES FOR EACH SUBJECT DURING THE DIFFERENT DIETARY PERIODS. In period II, group A subjects received 475 mg, group B 950 mg, and group C 1,425 mg of egg yolk cholesterol. In period IV, group A subjects received 1,200 mg of crystalline cholesterol, group B 2,400 mg, and group C 3,600 mg.

was determined by the method of Pihl (7). Phospholipid (5) and triglyceride (6) were determined on a chloroform-methanol extract. Values per 100 g of egg yolk were: cholesterol 1,370 mg, phospholipid 9.44 g, and triglyceride 25.18 g. These corresponded with previously reported analyses of egg yolk (8, 9).

Clinical course. The experimental subjects remained in good health for the duration of the study. They were examined daily; they took a constant amount of exercise each day; adherence to and tolerance of the formula were excellent. As might be expected with a 3,000 calorie intake, the smaller subjects (2, 3, 4 and 5) gained weight moderately during each dietary period. The total gain for the 12 weeks was from 4 to 13 pounds in these subjects. The larger subjects (1 and 6) had no weight changes.

RESULTS

The serum cholesterol, phospholipid and triglyceride values for each of the six subjects during the different dietary periods are illustrated in Figure 1 and Table II. These normal men, consuming a general diet, had a wide range of serum cholesterol concentration, varying from 198 to 295 mg per 100 ml (mean 249). After 1.5 weeks of the cholesterol-free formula in period I, the serum cholesterol of all men declined considerably. The downward trend continued, and after 3 weeks the mean serum cholesterol reached 191, or a mean change of − 58 mg per 100 ml ($p < 0.01$).

The addition of cholesterol in the form of egg yolk during period II for 3 weeks caused an increase in the serum cholesterol of each subject. The mean increase was 69, or from 191 to 260 mg per 100 ml (Figure 2). For the group, this change was significant ($p < 0.001$). The different amounts of dietary cholesterol led to similar responses. For example, the addition of 475 mg of cholesterol to the diet produced a mean increase of 66 mg per 100 ml in the serum cholesterol for Subjects 1 and 2 of group A. Twice

TABLE II

Mean serum lipid values for the different dietary periods*

		Period I			Period II			Period III			Period IV		
	Subject	Initial	Final	Change	Initial	Final	Change	Initial	Final	Change	Initial	Final	Change
Cholesterol	1	223	155	−68	155	208	+53	208	166	−42	166	176	+10
	2	295	201	−94	201	280	+79	280	240	−40	240	261	+21
	3	288	233	−55	233	297	+64	297	250	−47	250	278	+28
	4	241	194	−47	194	257	+63	257	181	−76	181	191	+10
	5	253	204	−49	204	282	+78	282	212	−70	212	233	+21
	6	198	164	−34	164	240	+76	240	185	−55	185	211	+26
	Mean	249	191	−58	191	260	+69	260	205	−55	205	224	+19
				SE 8.5			SE 4.3			SE 6.1			SE 3.2
				$p<0.01$			$p<0.001$			$p<0.001$			$p<0.01$
Phospholipid	1	181	108	−73	108	145	+33	145	125	−20	125	133	+ 8
	2	214	150	−64	150	208	+58	208	170	−38	170	191	+21
	3	236	194	−42	194	244	+50	244	180	−64	180	211	+31
	4	194	164	−30	164	203	+39	203	144	−59	144	156	+12
	5	185	155	−30	155	183	+27	183	155	−27	155	177	+22
	6	177	152	−25	152	193	+41	193	138	−55	138	178	+40
	Mean	198	154	−44	154	196	+42	196	152	−44	152	174	+22
				SE 8.2			SE 4.3			SE 7.3			SE 4.8
				$p<0.01$			$p<0.001$			$p<0.01$			$p<0.01$
Triglyceride	1	86	45	−41	45	53	+ 8	53	67	+14	67	48	−19
	2	118	78	−40	78	130	+52	130	128	− 2	128	125	− 5
	3	127	72	−55	72	101	+29	101	104	+ 3	104	91	−31
	4	103	66	−37	66	93	+27	93	72	−21	72	71	− 1
	5	183	109	−74	109	147	+38	147	118	−29	118	115	− 3
	6	91	58	−33	58	103	+45	103	102	− 1	102	119	+17
	Mean	118	71	−47	71	104	+33	104	98	− 6	98	95	− 4
				SE 6.3			SE 6.3			SE 6.5			SE 5.0
				$p<0.001$			$p<0.01$			$p<0.5$			$p<0.5$

* Computed on the basis of determinations made during the last week of each dietary period (mg per 100 ml). The initial values for period I are those obtained during the week of the general diet directly preceding period I. SE = the standard error of the mean or $SE = SD/\sqrt{N}$.

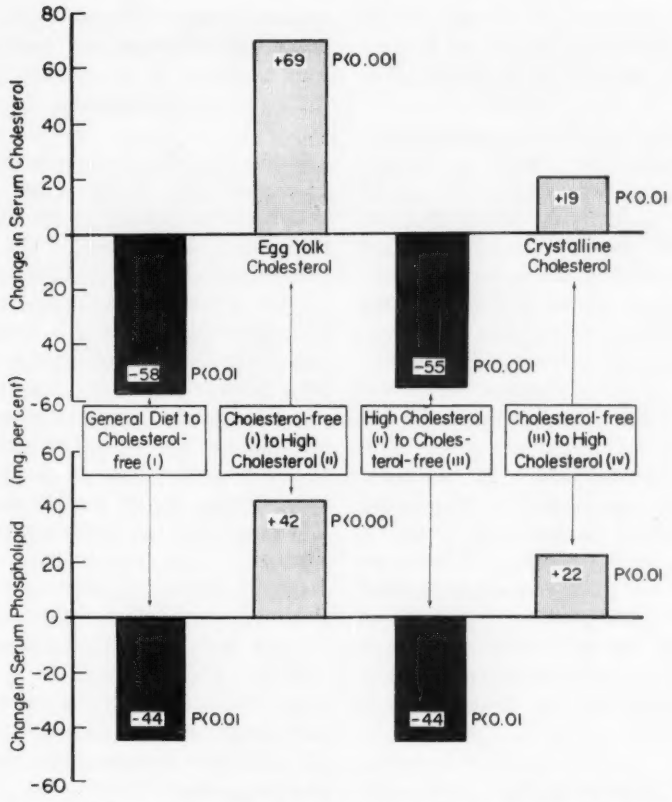

FIG. 2. THE CHANGES IN SERUM CHOLESTEROL AND PHOSPHOLIPID WHICH OCCURRED AS THE DIET WAS ALTERED FROM ONE PERIOD TO ANOTHER. Mean values for the six subjects are given.

this amount of dietary cholesterol led to no greater serum cholesterol increase (mean 64 mg per 100 ml) for Subjects 3 and 4 of group B. Dietary cholesterol of 1,435 mg given to Subjects 5 and 6 of group C led only to a slightly greater serum cholesterol change (mean 77 mg per 100 ml).

The removal of cholesterol from the diet (period III) led to a considerable decrease in the serum cholesterol of each subject. After 3 weeks of this diet the mean decrease was − 55, or from 260 to 205 mg per 100 ml and was significant ($p < 0.001$).

Crystalline cholesterol was then added to the diets for 3 weeks (period IV). The serum cholesterol concentration for each subject increased somewhat but much less than when cholesterol from egg yolk was given. The mean change was 19 mg per 100 ml ($p < 0.01$). The larger amounts of crystalline cholesterol did not seem to have greater effects on the serum cholesterol.

The serum phospholipid concentration for each subject changed in direction and amount as did the serum cholesterol for the different dietary periods. Initially, the consumption of the cholesterol-free diet resulted in a mean decline of 44 mg per 100 ml ($p< 0.01$). The high cholesterol diet of egg yolk (period II) caused a rise in the mean serum phospholipid of 42 mg per 100 ml ($p < 0.001$). The serum phospholipid increases were similar for groups A, B and C despite the feeding of different amounts of egg yolk cholesterol. The removal of egg yolk from the diet brought about a mean decrease in the serum phospholipid of 44 mg per 100 ml ($p < 0.001$). The mean serum phospholipid increased 22 mg per 100 ml ($p < 0.01$) when crystalline choles-

terol was added to the diets. The larger quantities of crystalline cholesterol appeared to produce a somewhat greater increase in the serum phospholipid than did the smaller quantities.

The serum triglyceride changes were variable and appeared unrelated to dietary cholesterol. The greatest shift was the mean decrease of 47 mg per 100 ml, which occurred when the diet was shifted from general to cholesterol-free (period I). Serum triglyceride increased when egg yolk cholesterol was added (period II) but was not affected by either the cholesterol-free diet of period III or the crystalline cholesterol of period IV.

The excretion of lipids in the stool was within normal limits, at or less than 5 per cent, for formulas with and without cholesterol. Stool fat increased, however, during period IV when the formulas contained large quantities of crystalline cholesterol. A typical example was Subject 5. He excreted 2.0 g of fat on the cholesterol-free diet and 5.2 g of fat while consuming the diet containing 3,600 mg of crystalline cholesterol per day. We infer that the bulk of this increase in lipid was unabsorbed dietary cholesterol, without the specific knowledge that the increase was in the sterol fraction.

DISCUSSION

In experimental animals, cholesterol is an important constituent of diets given to produce hypercholesterolemia and ultimately atherosclerosis (10). In man, on the contrary, most investigators have concluded that dietary cholesterol has little or no influence upon the serum cholesterol concentration (11, 12). Recent publications eliminate or omit any consideration of dietary cholesterol in the treatment and prevention of human hypercholesterolemia and atherosclerosis (13, 14). Emphasis has been placed upon dietary fat and protein and nondietary factors such as heredity, exercise and smoking.

A large number of experimental observations from Anitschkow's important report in 1913 to the present day has strengthened the concept that cholesterol in the diet is almost invariably essential for the production of atherosclerosis in animals. These species have ranged from the chicken to the monkey. Recently, Taylor, Cox, Counts and Yogi (15) have linked more closely human and experimental atherosclerosis. Extensive coronary atherosclerosis and myocardial infarction were produced in the monkey by the long-term feeding of a high cholesterol, 22 per cent butterfat diet.

In the present study, six healthy men had significant changes in the serum concentration of cholesterol in response to variations in their dietary intake of cholesterol. The total number of calories, the character and the amount of fat, the amount of protein and the amount of physical activity were maintained at a constant level, while only the amount of cholesterol in the diet was varied. Dietary cholesterol was first provided as egg yolk after the men had consumed a cholesterol-free diet for 3 weeks. The high cholesterol diet caused a prompt rise of the serum cholesterol concentration of each man by the end of 1 week. The removal of egg yolk cholesterol provided additional evidence of its influence upon serum cholesterol. The serum cholesterol of each man then decreased after 4 days of the cholesterol-free diet. For the next 2.5 weeks it continued to fall additionally. The changes in the serum phospholipid concentration of each man also correlated well with the presence or absence of cholesterol in the diet. In our previous study the high cholesterol and cholesterol-free diets were given for a longer period. The serum cholesterol and phospholipid changes induced by these diets were maintained for the entire 11 weeks of study (2).

Six egg yolks with a cholesterol content of 1,425 mg did not cause a greater rise in the serum cholesterol than did two egg yolks with one-third as much cholesterol. The review of Cook (16) and the work of Karvinen, Lin and Ivy (17) suggest that the human gastrointestinal tract has a limited capacity for cholesterol absorption. Perhaps all cholesterol above a certain amount is unabsorbed and passes out in the stool. The epidemiological study of Keys and co-workers provides data giving a similar line of evidence (11). Two large groups of Minnesota businessmen had similar serum cholesterol concentrations despite different cholesterol intakes of 401 and 1,010 mg per day.

Crystalline cholesterol had a lesser effect upon the serum cholesterol and phospholipid concentrations than did egg yolk cholesterol despite the

greater amounts of crystalline cholesterol which were consumed. The increased stool lipid during the period of the crystalline cholesterol formula suggested that cholesterol in this form might be poorly absorbed. Cook, Edwards and Riddell found that intestinal absorption of egg yolk cholesterol was four times more complete than was crystalline cholesterol dissolved in olive oil (18). The more effective absorption of egg yolk cholesterol may result from its dispersed state in lipoprotein complexes in the yolk.

The mechanism whereby dietary cholesterol augments the serum cholesterol concentration may be a matter of simple addition or it may be mediated through a more complex mechanism. First, after intestinal absorption, dietary cholesterol is transported in the chyle and later in the blood largely as a constituent of chylomicrons. When chylomicrons circulate in the blood, chylomicron cholesterol contributes to the total serum cholesterol (19). The continuance of chylomicron cholesterol in the blood probably depends upon the rapidity of chylomicron removal and hydrolysis by the liver and other tissues, but this is uncertain (20, 21). Second, cholesterol derived from the diet may recirculate as a higher density lipoprotein after chylomicron dissolution (21). In the rat, dietary cholesterol contributes as much as 90 per cent of the total serum cholesterol (22). In men given radio-labeled cholesterol, from 24 to 31 per cent of the serum cholesterol is derived from diet (23). The component of serum cholesterol not derived from the diet is of endogenous origin (22, 24).

Previous studies involving the effects of egg feeding upon the serum cholesterol concentration (25–27) are difficult to interpret, since they did not balance all of the variables between the control and experimental diets (2). Usually the egg yolk was an addition to a general diet and thereby caused a change in the fat content, fatty acid composition, calories and protein of the diet.

In the present study the egg yolk was not an addition. It was incorporated completely into the framework of the diet so that the cholesterol-free and egg yolk cholesterol formulas were identical in calories, protein and total fat. In particular, the differences in fatty acid composition between the two diets were negligible. The small quantity of arachidonic fatty acid fed only during the period of the egg yolk cholesterol formula might be expected to lower slightly the serum cholesterol, according to the studies of Nothman and Proger (28).

Cholesterol appears to be the necessary constituent of egg yolk that affects the serum cholesterol concentration (2). The phosphatide content of egg yolk would not be expected to have a direct hypercholesterolemic effect. Other phosphatides added to the diet have induced lowering of the serum cholesterol (29). It is not known whether egg yolk phosphatide enhances gastrointestinal absorption of egg yolk cholesterol. Bronte-Stewart has emphasized that egg yolk lipid elevates the serum cholesterol levels higher and more rapidly than does any other fat on a gram-for-gram basis (30). He has given evidence that both cholesterol and fat of a relatively high iodine value (i.e., 70) must be in the intestinal tract at the same time in order to achieve the hypercholesterolemic effects of cholesterol ingestion.

Beveridge, Connell, Mayer and Haust (31) recently reported that dietary cholesterol consumed with a butterfat fraction (30 per cent of the total calories) caused an increase in the serum cholesterol concentration. Their subjects consumed a fat-free formula for 8 days and for the next 8 days received a butterfat fraction plus cholesterol in amounts varying from 13 to 4,500 mg. In this study two variables in the diet, total fat and cholesterol, were altered. The serum cholesterol might be expected to increase when the fat alone was added to the diet. Nonetheless, the serum cholesterol concentration increased further by a significant amount when quantities up to 634 mg of cholesterol were added to the diets of various groups.

The observations of the present study are compatible with the thesis that dietary cholesterol has an important effect upon the serum lipids in human beings (2). This does not deny the effects of the other factors, especially the influence of polyunsaturated fats in the diet. It is of interest that in this study a decided increase in the serum cholesterol concentration occurred from the ingestion of dietary cholesterol at the same time that a considerable amount of polyunsaturated fat (35 g) was present in the diet. The clinical implications of such a conclusion must await the results of further investigation. The possible role of

dietary cholesterol should be considered in the design of diets given for the treatment and prevention of hypercholesterolemia.

SUMMARY

1. Six healthy men consumed in sequence a cholesterol-free diet, a diet containing cholesterol from egg yolk, a cholesterol-free diet, and a diet with added crystalline cholesterol. These diets were identical in calories, fat, protein, carbohydrate, minerals and vitamins. The fatty acid composition of the fat was also equivalent for all diets.

2. The addition of dietary cholesterol in the form of egg yolk caused a significant increase in the concentration of cholesterol and phospholipid in the serum. The serum cholesterol and phospholipid decreased greatly when egg yolk cholesterol was removed from the diet. Amounts of dietary cholesterol from 475 to 1,425 mg per day (two to six egg yolks) produced similar effects. These amounts are within the average range of cholesterol consumption in the United States.

3. When crystalline cholesterol was added to the cholesterol-free diet, less striking but significant increases in the serum cholesterol and phospholipid occurred.

4. These studies suggest that dietary cholesterol has a significant effect upon the serum cholesterol concentration in human beings.

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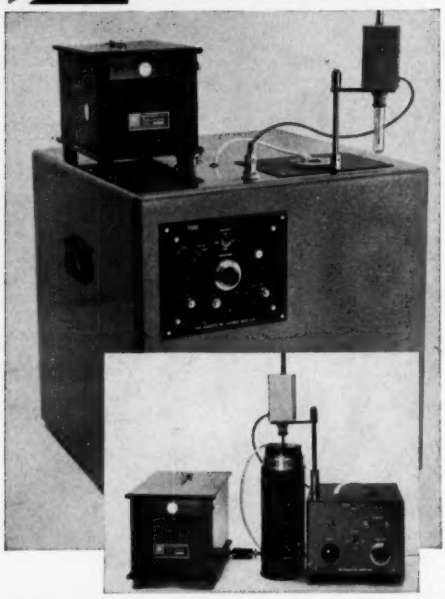

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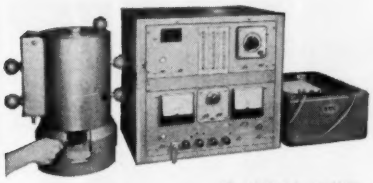

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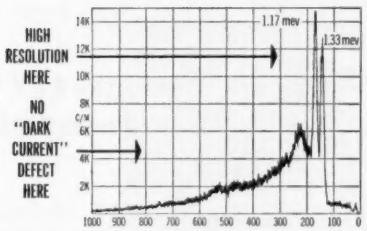
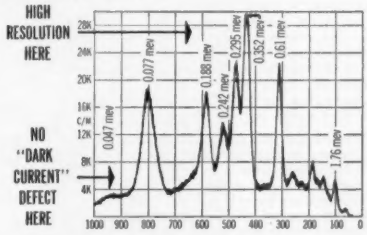


Actual spectrogram of Cobalt-60 made with a GSS-1 gamma spectrometer system. Window No. 3. Time constant: 2 sec.



Radium, after four runs over 20 hour period without instrument adjustment. Range 30,000 C/M. Time constant: 3 sec.

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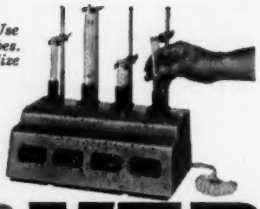

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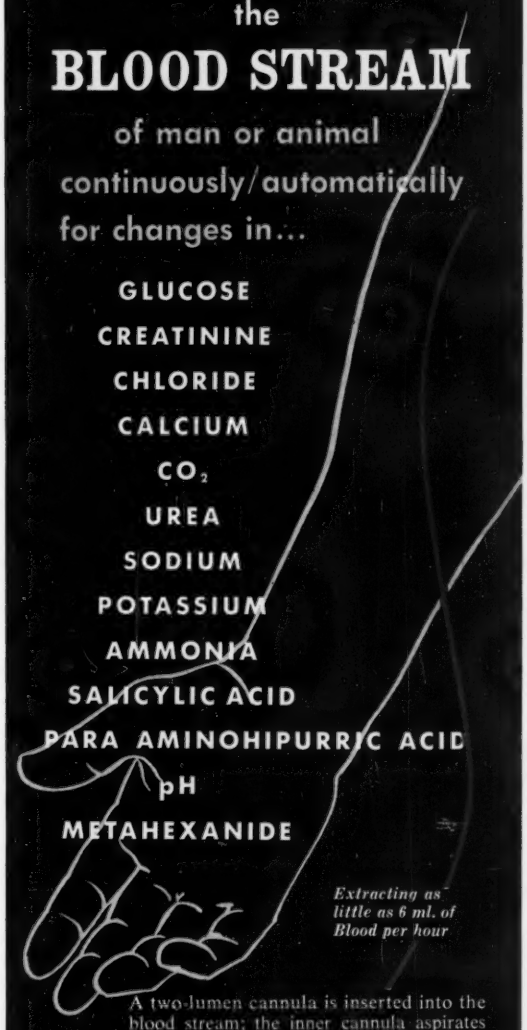

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May, 1961

Vol. VI, No. 5

Editorial

Etiocholanolone, Steroides Pyrogenes et "Maladie Periodique. E.-E. BAULIEU.

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Etude Immuno-Electrophoretique du Serum des Prematures. M. FOUCAUT et M. GOUDEMAND.

Etude Quantitative des Gamma-Globulines Seriques chez des Souris Irradiees et Restaurees par des Cellules Hematopoietiques Isologues ou Homologues. J.-L. AMIEL, G. MATHÉ et M. PAYS.

Etude Biochimique d'un Cas de Carcinoidose. A. CRASTES de PAULET, O. FLANDRE et I. COHEN.

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Technical Note

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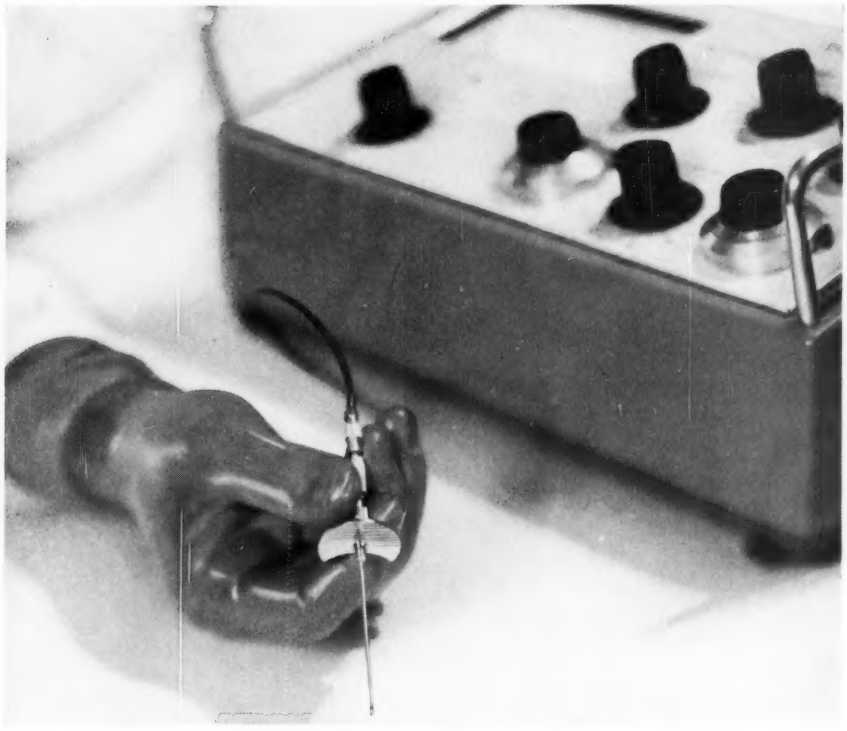

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